The influence of membrane components on the uptake of peptides by gram-negative bacteria

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by

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ABSTRACT

The number and specificities of peptide transport systems in Escherichia coli are investigated using direct and sensitive fluorescence assays and transport-deficient mutants isolated as resistant to peptide mimetic antibiotics. It is shown that there are three peptide permeases of which the oligopeptide permease (Opp) is confirmed as being capable of transporting a wide range of di- and oligopeptides and the dipeptide permease (Dpp) and third peptide permease (Opt) are both shown to have wider specificities than has been previously reported. The Opt is shown to be energised directly by phosphate bond energy and is coded for by a gene mapping between 84 and 88 min.

The applicability of monitoring the kinetics of peptide uptake as a means of measuring outer membrane permeability is studied. Using this method with wild type and outer membrane protein deficient strains, the porins are shown to be the major route for peptide diffusion across the outer membrane. The advantages of using this approach, and the possible mechanisms of the decrease in permeability caused by loss of OmpA, are discussed.

Intact peptide uptake is shown in Pseudomonas aeruginosa. Uptake appears to be constitutive, although slow compared with that in other bacteria, and distinct from amino acid transport. There are probably two transport systems, analogous to the Dpp and Opp of E. coli, both coupled to phosphate bond energy and capable of transporting a range of di- and oligopeptides.

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CONTENTS

1 INTRODUCTION

1.1 INTRODUCTION

For a long time peptides have been known to be nutritionally valuable for a wide range of microorganisms. Many commercially available growth media contain peptones (see for example, Difco Manual, 9th edition, 1953) which consist largely of a mixture of small peptides and amino acids. Only in the last 20 years however, since defined small peptides have become available, have peptide transport systems been studied in detail. Intact peptide uptake has now been demonstrated in a variety of organisms including: prokaryotes, eukaryotic microorganisms, protozoans, mammalian organs and plants (for reviews see Payne, 1980; Becker and Naider, 1980; Wolfinbarger, 1980; Rasmussen and Zdanowski 1980; Matthews, 1975; Higgins and Payne, 1980; Matthews and Payne, 1980).

Much of the work on peptide transport in microorganisms has centred on the Enterobacteriaceae and on Escherichia coli in particular. A lack of suitable assays for peptide transport has greatly hindered work on peptide transport so that not even the transport systems of E. coli are well

characterised. Recent advances in assay techniques (Payne and Bell, 1979; Nisbet and Payne 1979a; Payne and Nisbet, 1981) have allowed transport systems to be directly investigated in detail. In the present work, these new techniques have been used to characterise the peptide transport systems of E. coli in more detail than was previously possible.

Recently there has been interest in the possibility of producing novel antibiotics using peptides to introduce otherwise impermeant toxic moieties into the cell. To design the most effective antibiotics, the peptide transport specificities of the target organism must first be well characterised. Pseudomonas aeruginosa is a clinically important organism and is resistant to most of the antibiotics currently in use so that for this organism in particular a new class of antibiotic agent, would be useful. It was with this in mind that an investigation of peptide transport in P. aeruginosa was also undertaken here.

Payne (1968) deduced, from the results of competition studies, that the upper size limit for peptide transport in E. coli was likely to be a property of the cell envelope rather than the peptide permease per se . At that time very little was known about the permeability properties of the cell envelope of Gram-negative bacteria. Since then there

has been considerable interest in this area and we now have a much better understanding of the function of the outer membrane. It seemed an opportune time, in the light of these recent advances, to investigate further the role of the outer membrane in peptide transport in E. coli. The effects of the loss of outer membrane proteins on peptide transport have been studied here.

In this introductory chapter the literature in the following areas is surveyed:

Section 1.2. The structure and function of the outer membrane of Gram negative bacteria.
Section 1.3. Peptide transport in microorganisms.
Section 1.4. Methods for studying peptide transport.
Section 1.5. Nitrogen metabolism in Pseudomonas aeruginosa.

1.2 STRUCTURE AND FUNCTION OF THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA

Introduction

Almost all bacteria have a lipid bilayer cytoplasmic membrane and a rigid peptidoglycan layer around their cells. Gram-negative bacteria also possess a second membrane outside the peptidoglycan layer, known as the outer membrane. In between the inner membrane and the outer membrane is an area called the periplasmic space (see Figure 1.1). Gram-negative bacteria are generally more resistant to detergents and hydrophobic antibiotics than are Gram-positive bacteria, which can largely be ascribed to the presence or absence respectively of the outer membrane. The relative impermeability of the outer membrane to certain molecules, yet free permeability to others e.g. small hydrophilic nutrient molecules, has lead to much recent interest in the structure and function of the outer membrane. A complete review of the investigations of the outer membrane is impossible in the space available, therefore it is proposed to concentrate mainly on the aspects most relevant to the work presented here. Further details of areas not covered here may be obtained from the recent reviews of outer membrane structure and function (Inouye, 1979; Lugtenberg, 1981; Lugtenberg and Van Alphen, 1983).

Cell envelope structure

The cytoplasmic membrane is mainly phospholipid and protein. It is involved in active transport of nutrients

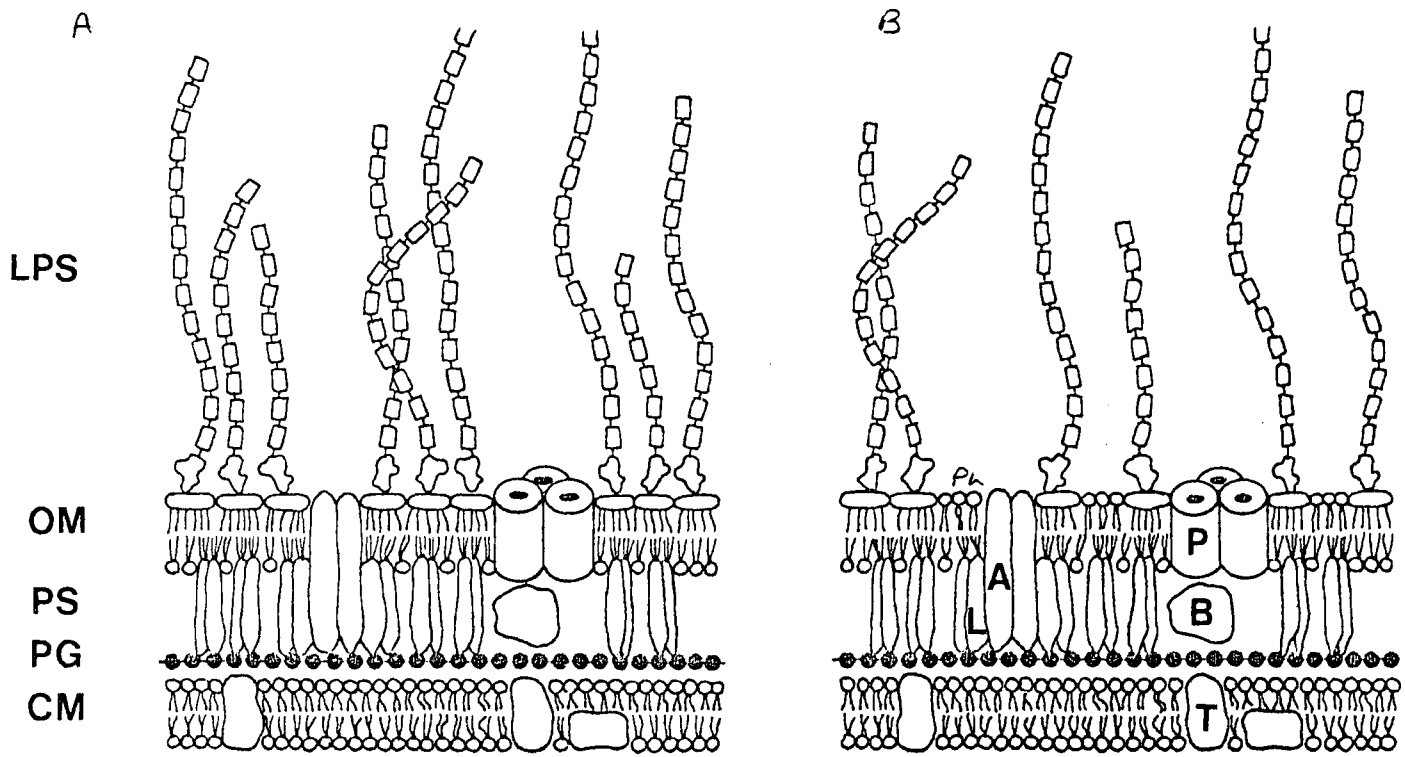


Figure 1.1 Gram-negative cell envelope structure (after Lugtenberg and Van Alphen, 1983). A represents an enterobacterial outer membrane with no outer leaflet phospholipid. B represents the outer membrane of a penicillin sensitive species e.g. Neisseria, where phospholipid is assumed to be present in the outer leaflet. Abbreviations: OM, outer membrane; CM, cytoplasmic membrane; PS periplasmic space; PG, peptidoglycan; LPS, lipopolysaccharide; Ph, phospholipid; P, porin protein; A, OmpA protein; L, lipoprotein; B, periplasmic binding protein; T, transport protein.

into the cell and also plays a role in the synthesis and export of phospholipids, peptidoglycan, lipopolysaccharide and periplasmic and outer membrane proteins.

The peptidoglycan is a network of chains of alternate N-acetylmuramic acid and N-acetylglucosamine residues, cross-linked to give the structure rigidity. The peptidoglycan is largely responsible for cell shape and allows the cell to grow in hypotonic environments where turgor pressure would otherwise cause cell lysis. In hypotonic conditions, the cytoplasmic membrane will be pushed up against the peptidoglycan by turgor pressure, and in hypertonic environments the cytoplasmic membrane will pull away from the peptidoglycan during plasmolysis. The peptidoglycan is linked to the outer membrane via proteins and helps to anchor the outer membrane in place.

The periplasmic space lies between the inner and outer membranes and may be as much as 40% of the total cell volume (Stock et al., 1977). The periplasmic space contains proteins and oligosaccharides. The proteins have a range of functions including, substrate binding proteins (essential for some active transport systems), enzymes for converting nutrient molecules into substrates for permeases and enzymes involved in cellular defence mechanisms e.g. B-lactamases. The oligosaccharides are probably involved in regulating the

cellular osmotic balance (Kennedy, 1982).

The outer membrane contains phospholipid, lipopolysaccharide (LPS), lipoprotein and protein. The lipoprotein anchors the outer membrane to the peptidoglycan. The outer membrane provides a barrier to detergents, enzymes and antibiotics in the environment and also prevents leakage of periplasmic proteins. The outer membrane is also involved in cell-cell interactions and host-cell interactions in pathogenic species and contains receptor sites for phages, etc. (Inouye, 1979).

There are several hundred so-called zones of adhesion between the inner and outer membranes (Bayer, 1979), which are believed to be involved in the translocation of outer membrane components from their site of synthesis on the inner membrane to their sites in the outer membrane.

Additional layers may be present outside the outer membrane e.g. a capsular layer of polysaccharide or an A-layer of repeated protein sub-units. These additional layers are probably involved either in protecting the cell against harmful environments or in adherence to host tissues (Lugtenberg and Van Alphen 1983). Flagellae and the sex pili (when present) are anchored in the cell envelope.

Outer membrane components

The development of techniques for separating the inner and outer membranes, by sucrose density gradient centrifugation (Miura and Mizushima, 1969) or by differential solubilisation in detergents (Schnaitman, 1970), allowed the constituents of the outer membrane to be analysed. The major constituents were shown to be highly variable (De Leij and Witholt, 1977; Schweizer *et al.*, 1976; Smit *et al.*, 1975; Gmeiner and Schlecht, 1979,1980) even between strains of *Escherichia coli*, although this may be due in part to different growth conditions and assay procedures. Most of the work in this area has been done on Enterobacteria.

Phospholipid

Over 90% of the phospholipid in the outer membrane is phosphatidylethanolamine (compared with 50-60% in the inner membrane), while the rest is a mixture of phosphatidylglycerol and diphosphatidylglycerol (Lugtenberg and Peters, 1976; Bayer, 1979). The phosphatidylethanolamine in the outer membrane contains more saturated fatty acids and fewer species with two unsaturated fatty acids than the phosphatidylethanolamine in the inner membrane (Lugtenberg and Peters, 1976; Ishinaga *et al.*, 1979). There is a rapid interchange between phospholipid in the inner and outer

membranes (Jones and Osborn, 1977a,b), but the asymmetry between the membranes is maintained. It has been suggested that this asymmetry is maintained because phosphatidylethanolamine forms stable bilayers with LPS (Fried and Rothfield, 1978). In Salmonella typhimurium it has been calculated that the phospholipid present is barely enough to fill one side of the lipid bilayer (Smit et al., 1975; Kamio and Nikaido, 1976). Several studies have shown that phospholipids are not accessible to exogenous agents (Tamaki et al., 1971; Tamaki and Matsuhashi, 1973; Wilkinson, 1972; Schindler and Teuber, 1978; Van Alphen et al., 1977). These experiments indicate that the phospholipid is almost entirely restricted to the inner leaflet of the outer membrane in E. coli and S. typhimurium. In other Gram-negative organisms that are sensitive to hydrophobic antibiotics, there may also be some phospholipid in the outer layer of the membrane (Lysko and Morse, 1981).

Lipopolysaccharide

LPS is only found in the outer membrane of Gram-negative bacteria (Muhlradt and Golecki, 1975). It is an amphipathic molecule consisting of a hydrophobic part called lipid A and a hydrophilic sugar chain which is often branched (a schematic representation of the structure of LPS is shown in Figure 1.2).

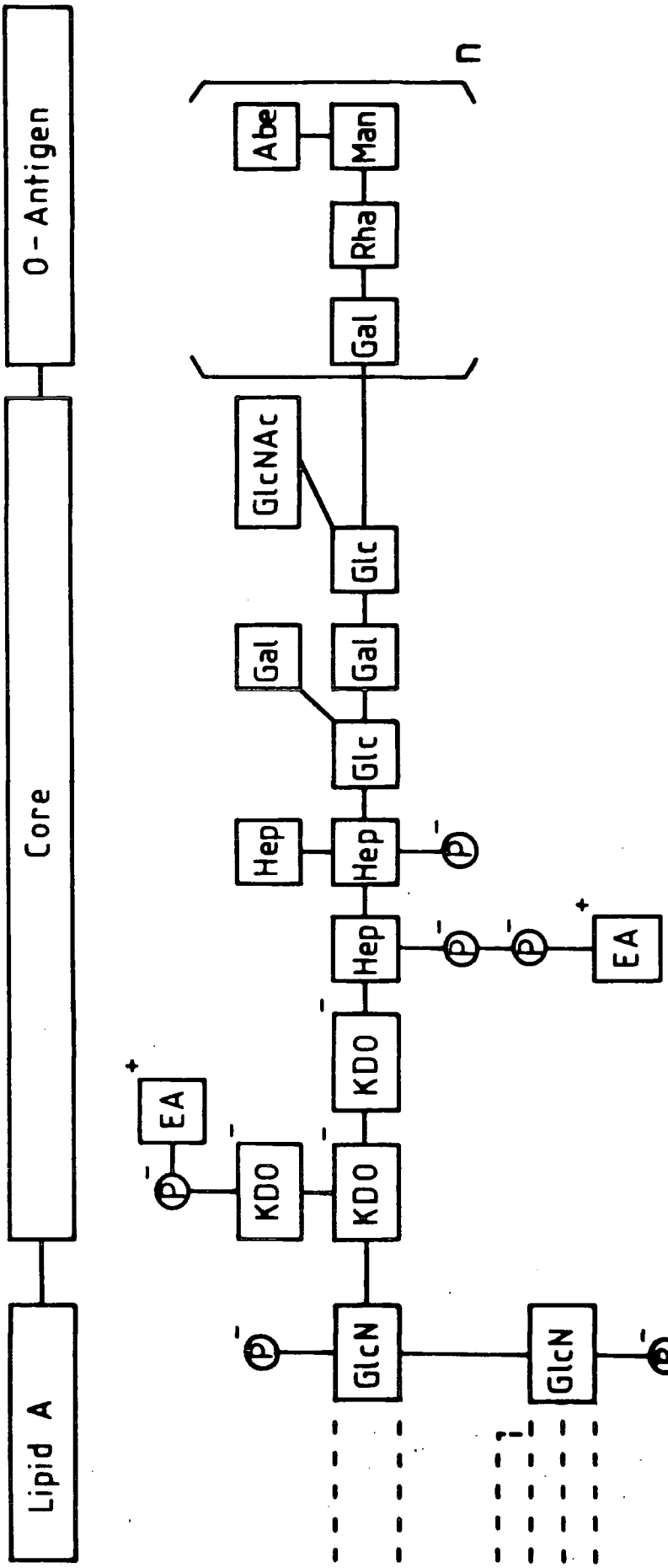


Figure 1.2 Structure of the lipopolysaccharide. Dashed lines represent fatty acyl chains. Abbreviations: EA, ethanolamine: Gal, galactose; Glc, glucose· GlcN, glucosamine; GlcNAc, N-acetyl glucosamine: Hep, heptose: KDO, 2-keto-3-deoxyoctonate; Man, mannose: P, phosphate: Rha, rhamnose. Abe, Abequose

Lipid A, is a highly conserved structure in the Enterobacteriaceae (Luderitz et al., 1971), being a glycolipid containing a β-(1-6) linked glucosamine disaccharide unit, with a phosphate in position 1 and a phosphate or pyrophosphate in position 4', and approximately 6 fatty acyl chains comprising predominantly 12 or 14 carbon atoms (Rietschel et al., 1972; Rosner et al., 1979; Wollenweber et al., 1982).

The polysaccharide chain, which is covalently attached to the lipid A base, consists of two parts, the core region, which is highly conserved amongst the Enterobacteriaceae, and the O-antigen which shows great diversity even among strains of the same species. The core region always contains the sugars 3-deoxy D-manno octulosonic acid and L-glycero D-manno heptose in Enterobacteriaceae. There are also several other sugars present e.g. glucose and galactose (Osborn, 1969; Luderitz et al., 1971). The O-antigen consists of units of 3-6 sugar residues with up to forty or more repeating units. A wide range of different and often charged sugars are found in the O-antigen, and the length of the chain can vary even among cells in the same culture (Goldman and Leive, 1980). For a more detailed account of the structure of the LPS see Galanos et al. (1977) or Lugtenberg and Van Alphen (1983).

Studies using mutants containing partially defective LPS have shown that LPS is mainly responsible for the impermeability of the outer membrane to hydrophobic molecules. The outer layer of the outer membrane is thought to consist entirely of LPS and protein in a 60:40 ratio (Lugtenberg and Van Alphen, 1983). Both the hydrophilic portion of proteins exposed at the cell surface and the highly charged core region of the LPS effectively prevent diffusion of hydrophobic molecules. In those Gram-negative organisms which are relatively permeable to hydrophobic molecules it has been suggested (Lysko and Morse, 1981) that there are also patches of phospholipid present in the outer layer and that this allows the diffusion of hydrophobic molecules.

Enterobacterial common antigen

Enterobacterial common antigen (ECA) is found in the outer membrane of most Enterobacteriaceae (Makela and Mayer, 1976). ECA is a polymer of N-acetyl D-glucosamine and D-mannosaminuronic acid partly esterified by palmitic acid (for reviews of the structure and function of ECA see Makela and Mayer, 1976; Mayer and Schmidt, 1979). ECA can either exist in a free form, or attached to LPS in a similar way to O-antigen.

Proteins

The outer membrane contains large numbers of relatively few protein species (major outer membrane proteins) and small numbers of a further 10-20 protein species (minor outer membrane proteins), although these terms can be misleading as under certain growth conditions the production of some minor proteins can be increased. The major proteins consist of the lipoprotein, the OmpA protein and the porin proteins. The minor proteins include specific pore proteins and receptor proteins for phages and colicins.

Very few enzymic activities have been reported to be associated with the outer membrane but those which have include: in E. coli, phospolipase, UDP-glucose hydrolase, monoacylglycerophosphoethanolamine acylase, protease IV and signal peptidase (Lugtenberg and Van Alphen, 1983), in Serratia marcescens, lipase, nuclease and protease activities have been found associated with the outer membrane (Braun and Schmitz, 1980; Winkler et al., 1978) although this may only be an intermediate location in protein excretion (Heller, 1979). Neisseria meningitidis outer membranes have been shown to contain tetramethylphenyldiamine oxidase activity (De Voe and Gilchrist, 1976).

Lipoprotein

The lipoprotein is the most abundant protein in the bacterial cell with approximately 7.5×10^5 copies present. It was first discovered by Braun and Rehn (1969) and is often known as Braun's lipoprotein. In E. coli the lipoprotein has a molecular weight of 7,200 and contains 58 amino acid residues. It is bound by the ε-amino group of its C-terminal lysine to the α-carboxyl group of every tenth to twelfth diaminopimelic acid residue of the peptidoglycan. The N-terminal glycerylcysteine residue has three fatty acid chains attached, two by ester linkages and one by an amide linkage. The lipoprotein does not contain histidine, tryptophan, glycine, proline or phenylalanine. Only one third of lipoprotein molecules are found bound to the peptidoglycan, the other two thirds being in a free form. Both forms have been isolated and sequenced (Braun and Bosch, 1972; Inouye et al., 1976). Purified lipoprotein is rich in α-helix and probably forms a helical rod structure in vivo (Inouye, 1974). For reviews of the structure of the lipoprotein see DiRienzo et al. (1978), and Inouye (1979).

The structure of the lipoprotein is highly conserved among the Enterobacteriaceae (Nakamura and Inouye, 1979, 1980) and is also found in Aeromonas salmonicida (Evenberg et al., unpublished data in Lugtenberg and Van Alphen, 1983), Pseudomonas aeruginosa (Mizuno and Kageyama, 1979a) and Rhodopseudomonas spheroides (Baumgartner et al., 1980),

although the lipoprotein in these species has no homology with that of the Enterobacteriaceae.

The structural gene for the lipoprotein (lpp) has been mapped at 36.3min and mutants defective in this gene have been isolated. These mutants leak periplasmic enzymes and produce outer membrane vesicles (Hirota et al. , 1977), implying that the lipoprotein plays a role in stabilising the outer membrane.

The lpp genes of E. coli K12 (Nakamura and Inouye, 1979), Serratia marcescens (Nakamura and Inouye, 1980) and Erwinia amylovora (Yamagata et al., 1981) have been cloned and sequenced and confirm the high level of conservation of the lipoprotein. Multiple copies of the lpp gene on plasmids result in a rise in the levels of free lipoprotein but do not affect the levels of bound lipoprotein (Lee et al., 1981), however the use of a high copy number plasmid produces lethal overproduction of lipoprotein (Lee et al., 1981).

McLachlen (1978) has proposed that the acyl chains of the lipoprotein are buried in the inner leaflet of the outer membrane while the C-terminal end is attached to the peptidoglycan. In cross-linking studies, free lipoprotein was shown to form dimers and to link with OmpA (Reithmeier and Bragg, 1977). It is not yet clear whether the

lipoprotein also interacts with the porin proteins. Spin-labelling and other studies provide evidence in favour of such an interaction although attempts to cross-link porin proteins and the lipoprotein have not been successful (for review see Inouye, 1979). However all the amino groups in porin proteins are probably located within the pore (Tokunaga et al., 1981) and are therefore unavailable for cross-linking. Thus it still remains an open question as to whether there may be an interaction between lipoprotein and porin proteins.

Recently a new group of lipoproteins has been discovered. These are loosely associated with the peptidoglycan but are not related to Braun's lipoprotein (Ichihara et al., 1981). This type of lipoprotein has also been found in Proteus mirabilis (Mizuno, 1979) and Pseudomonas aeruginosa (Mizuno, 1979; Mizuno and Kageyama, 1979a).

OmpA protein

The OmpA protein has a molecular weight of 35,000 and contains 325 amino acid residues (Chen et al., 1980). It is heat modifiable in that when run on polyacrylamide gels it has an apparent molecular weight of 28,000 in the non-denatured form and 35,000 in the heat-modified form (Schnaitman, 1973; Reithmeier and Bragg, 1974). This

difference is probably due to the high content of β-structure and excessive binding of SDS to the non-denatured form (Heller, 1978; Chen et al., 1980). Up to 25% of the lysine residues in OmpA are oxidised to allysine (α-amino adipic acid semialdehyde) and it has been suggested that these residues could cross-link to the peptidoglycan layer (Diedrich and Schnaitman, 1978).

The structural gene (ompA) has been mapped at 21.5min on the E. coli K12 chromosome (Henning et al., 1976). Mutants defective in ompA have been selected by using resistance to phages K3 or TuII or colicin L (Chai and Foulds, 1974). Mutants defective in ompA have increased amounts of phospholipids (Van Alphen et al., 1977) and porins (Henning and Haller, 1975) and are also defective in F-pilus mediated conjugation (Skurray et al., 1974). Expression of ompA on a low copy number plasmid can increase the levels of OmpA protein up to twofold with a concomitant loss of other outer membrane proteins (Henning et al., 1979). Higher levels of OmpA production are lethal (Henning et al., 1979).

Studies using proteolytic enzymes and cloned gene fragments show that OmpA protein consists of two domains. The N-terminal 180 residues are thought to be located in the outer membrane with the remaining residues in the periplasmic space (Schweizer et al., 1978).

Mutants defective in both OmpA production and lipoprotein production, lose their cell shape and become spherical(Sonntag et al., 1978), indicating that both these proteins play a role in maintaining outer membrane stability, probably by binding it to the rigid peptidoglycan layer.

Cross-linking studies have shown that OmpA protein forms oligomers with itself, dimers with the free form lipoprotein (Reithmeier and Bragg, 1977) and also interacts with the peptidoglycan (Hall and Silhavy, 1979). The OmpA protein is also present at the cell surface as it acts as a receptor for phages and colicins (Van Alphen et al., 1977). Therefore it is concluded that OmpA protein extends right through the outer membrane and periplasmic space.

Porin proteins

The porin proteins (also known as matrix proteins or peptidoglycan-associated proteins) are a family of related proteins which form general diffusion pores. When cell envelopes are fractionated, the porin proteins are usually found associated with a lipoprotein-peptidoglycan complex, unless high temperatures or high salt concentrations are used when the porin proteins can be separated from this complex (Rosenbusch, 1974). It was also noted during these studies that the lipoprotein-peptidoglycan complex formed a

lattice-like structure in the presence of porin protein but not in their absence (Rosenbusch, 1974). These observations form the basis for the alternative names which were used in early work with the porin proteins.

The porins have molecular weights of 35,000-40,000 and have been isolated from many Enterobacteriaceae (Lugtenberg et al., 1977) and Pseudomonas aeruginosa (Hancock et al., 1981). All the porin proteins of the Enterobacteriaceae are highly conserved as shown by their reactions with antibodies raised against another porin (Hofstra and Dankert, 1979, 1980), and the homology between the DNA sequences of their structural genes (Mizuno et al., 1982b).

These proteins have been most extensively studied in E. coli where there are normally two porin species constitutively produced, OmpC and OmpF. In addition to these, there are also several other porin types produced but only under certain circumstances. PhoE production is induced by phosphate limitation (Overbeeke and Lugtenberg, 1980), Lc protein is only produced when cells undergo lysogenic infection with phage PA2 (Lee and Schnaitman, 1980), protein K is strongly correlated with encapsulation (Paakkanen et al., 1979) and a new membrane protein (NmpC) appears in some porin defective mutants (Lee et al., 1979). S. typhimurium constitutively produces three porins OmpC, OmpF and

OmpD,whereas *P. aeruginosa* constitutively produces protein F.

The proposed role of the porins in facilitating diffusion was indicated by experiments in which various outer membrane components were inserted into liposomes. Only the peptidoglycan-associated proteins were able to increase the permeability of liposomes towards small, hydrophilic solutes (Nakae, 1976). The fact that when the porins were inserted into the liposomes they displayed a similar upper size limit for molecules (approximately 600 molecular weight) to that of wild type cells provided evidence that it was these proteins which were largely responsible for solute penetration across the outer membrane. Further evidence on this point came from studies of the increase in conductance of black lipid membranes upon insertion of porins (Benz *et al.*, 1978) and the measurement of penetration rates across the outer membrane in porin-deficient mutants (Zimmermann and Rosselet, 1977; Van Alphen *et al.*, 1978a). There have been many studies of the porin pores using these techniques to elucidate the specificities and characteristics of each pore type (for review see Lugtenberg and Van Alphen, 1983) but, despite these studies, these features remain unclear.

In *E. coli* the pores formed by OmpC, OmpF and PhoE have slightly different diameters of 1.3nm, 1.4nm and 1.2nm respectively, (estimated by Benz and Hancock, 1981) or

0.54nm, 0.58nm and 0.53nm respectively (estimated by Nikaido and Rosenberg, 1983). In S. typhimurium however it appears that all three pores have a diameter of 1.4nm (Benz et al., 1980). The diameter of the pore formed by protein F in P. aeruginosa has been calculated at 2.2nm (Benz and Hancock, 1981). It has been suggested that molecules with molecular weights up to 6,000 can penetrate the outer membrane of P. aeruginosa and that such large pores might be more representative of most pores than the narrow pores in the Enterobacteriaceae (Hancock and Nikaido, 1978). In an attempt to reconcile the apparently large pore size of P. aeruginosa with its undoubted resistance to many antibiotics, it has been suggested that there is only a small proportion of these pores in an open configuration (Benz and Hancock, 1981) A recent report however, claims that there is an exclusion limit for molecules of molecular weight around 400 in P. aeruginosa (Caulcott et al., 1984). Preliminary evidence indicates that Neisseria gonorrheae also possesses wider pores than E. coli or S. typhimurium (Douglas et al., 1981). Clearly this is an area which requires further study.

Although originally proposed as being non-specific diffusion pores (Nikaido, 1979), it has become apparent that the rate of diffusion through each pore is affected by several parameters e.g. electrical charge, hydrophobicity and size. In E. coli, the PhoE pore preferentially transports

anionic molecules (Overbeeke and Lugtenberg, 1982; Nikaido et al., 1983) whereas with OmpF and OmpC pores a positive charge enhances diffusion. Hydrophilic molecules generally diffuse through porins better than hydrophobic molecules, as might be expected for aqueous pores (Nikaido et al., 1983), and smaller molecules diffuse more quickly than larger molecules, as one might also expect (Nikaido and Rosenberg, 1981). The OmpF pore tends to be more important in the diffusion of many molecules than the OmpC pore, partly, at least because of its larger diameter (Nikaido and Rosenberg, 1983) although additional effects of pore structure other than diameter cannot yet be ruled out. The specificity of the PhoE pore for anions might be expected as it is induced under conditions of phosphate limitation and presumably is designed to act as an efficient pore for phosphate ions and phosphate containing molecules.

Freeze-fracture studies indicate the presence of porin-LPS complexes in vivo and the pore-forming activity of all the porin proteins is dependent on the presence of LPS (Van Alphen et al., 1978b). The porin trimers are formed exclusively from the same pore type, with each monomer forming its own pore (Nakae et al., 1979). The porins cannot be cross-linked either to the peptidoglycan or to the lipoprotein, although the fact that porins are usually isolated associated in a complex with both these components

indicates that there is some interaction *in vivo*.

The genetics of porin production are complex, and although they have been intensively studied recently, they are not yet clearly understood. In *E. coli* the PhoE porin is part of the *pho* regulon at 6min on the chromosome (Tommassen and Lugtenberg, 1981). Transcription of this regulon is induced by growth in limiting phosphate concentrations (Overbeeke and Lugtenberg, 1980). The producton of OmpF and OmpC is controlled by three loci, *ompF* (21min), *ompC* (47min) and *ompB* (74min). The *ompF* and *ompC* loci contain the structural genes for OmpF and OmpC porins respectively (Reeves, 1979). The *ompB* locus consists of two genes *ompR* and *envZ* which are regulatory (Hall and Silhavy, 1979, 1981). Although both OmpC and OmpF are produced constitutively, the relative proportions of each porin in the outer membrane can vary widely depending on the culture conditions used. The changes in the level of one porin are compensated for by a reciprocal change in the other porin so that the overall level of porin remains approximately constant (Lugtenberg *et al.*, 1976). OmpF is preferentially produced under conditions of low osmolarity or high cAMP (Kawaji *et al.*, 1979; Scott and Harwood, 1980) while OmpC levels are raised in media with high osmolarity. This control has been shown to occur at the transcriptional level (Hall and Silhavy, 1979, 1981).

Genetic studies of the ompB locus have shown that mutants in ompR can show either an OmpF OmpC or an OmpF OmpC phenotype, while envZ mutants show an OmpF OmpC phenotype. Mutants in envZ also display pleiotropic effects, where the levels of several other periplasmic and outer membrane proteins are reduced (Lundrigen and Earhart, 1981). Hall and Silhavy (1981) proposed that the envZ product is an envelope protein which "senses" conditions in the external environment and regulates an equilibrium between two forms of the ompR product which in turn positively regulates ompF and ompC transcription. Cloning and sequencing of both ompR and envZ have shown that the OmpR protein has homology with DNA binding proteins and that the EnvZ protein may be membrane bound (Mizuno et al., 1982a,b). Recently Ozawa and Mizushima (1983) have reported that OmpC expression depends only on OmpF production and is independent of medium osmolarity in ompF mutants, whereas OmpF expression was dependent on medium composition in both ompC and ompC mutants. This is in contradiction to the finding of Morona and Reeves (1982) who reported that the production of both OmpF and OmpC was independent of medium composition in the absence of the other. How these differing results will be explained remains to be seen.

Minor proteins

Among the minor proteins found in the outer membrane are components of uptake systems for maltodextrins, nucleosides, vitamin B12 and iron. There are also several other proteins present to which no function has yet been ascribed. Many of these proteins act as receptors for phages and colicins and it is often the occurrence of phage and colicin resistant mutants which has led to the elucidation of the roles of these proteins. For a review of these functions see Konisky (1979).

The LamB protein, which is induced in the presence of maltose, is the receptor for phage lambda. It forms a pore largely specific for the uptake of maltodextrins (Szmelcman et al., 1976). The specificity of the pore has been shown to be dependent on the presence of the periplasmic maltose binding protein, so that in the absence of this binding protein, it acts as a general diffusion pore (Wandersman et al., 1979). The LamB pore is associated in trimers in its active form (Palva and Westerman, 1979) with each pore having a diameter of 1.5nm, which is slightly larger than the porin pores (Boehler-Kohler et al., 1979). The lamB gene has been sequenced and the polypeptide predicted from this sequence contains 421 amino acid residues with a molecular weight of 47,400 (Clement and Hoffnung, 1981).

The phage T6 receptor (Tsx protein) is a protein with a molecular weight of 26,000 (Manning and Reeves, 1978) coded for by the *tsx* gene at 9.2min. The protein is involved in the uptake of nucleosides and deoxynucleotides (except cytidine and deoxycytidine) and is catabolite repressible (Manning and Reeves, 1978). The Tsx protein has also been shown to allow the diffusion of serine, glycine and phenylalanine but not glucose or arginine (Heuzenroeder and Reeves, 1981). The solute specificities of the Tsx pore are as yet unclear.

Bacteria have developed several systems for scavenging the very low levels of soluble ferric iron in the environment. Iron chelators known as siderophores are used to complex ferric ions which are then taken up into the cell via outer membrane receptors and high affinity transport systems. Five outer membrane proteins have been shown to be involved in iron uptake in *E. coli* K12. The FepA protein is a protein of molecular weight 81,000 coded for by the *fep* gene at 13min. It is a receptor for colicins B and D, is induced by limiting iron concentrations and acts as a receptor for ferric enterochelin (Hantke, 1981). The FhuA or TonA protein (molecular weight 78,000) is coded for by the *fhuA* gene at 3.4min and acts as a receptor for phages T1, T5 and O80 and colicin M. FhuA is a receptor for ferrichrome and is also induced under conditions of iron limitation (Kadner *et al.*,

1980). The FecA protein (molecular weight 80,500) is co-induced with the ferric-citrate transport system in the presence of citrate. The FecA protein is a receptor for ferric citrate and is coded for by the fecA gene at 7min (Wagegg and Braun, 1981). The remaining two proteins, Cir (colicin I receptor) and the 83K protein are both induced under limiting-iron conditions, however they have not yet been shown to act as receptors for any iron complex (Konisky, 1979). The Cir protein (molecular weight 74,000) is coded for by the cir gene at 44min. The gene coding for the 83K protein has not yet been identified.

The BtuB protein which is a pore specific for vitamin B12, has a molecular weight of 60,000 and is induced under conditions of B12 limitation. It is a receptor for phage BF23 and the E-colicins and is coded for by the gene btuB at 89min (Konisky, 1979)

The proteins without identified function include, protein a (molecular weight 40,000) which may be a signal peptidase (Fiss et al., 1979), protein III (molecular weight 17,000) which has 10^4-10^5 copies per cell and may be essential, as mutants lacking it have never been isolated (Lugtenberg and Van Alphen, 1983). There are also two proteins of molecular weight 15,000 and 19,000 which are induced under conditions of low sulphate concentration (Lugtenberg and Van Alphen,

1983).

1.3 PEPTIDE TRANSPORT IN MICROORGANISMS

Introduction

Although peptides have been used as bacterial nutrients for over 100 years, little, if anything, was known of the mechanisms by which organisms utilised peptides until less than forty years ago. Early experiments from 1945 onwards indicated that peptides could act as an amino acid source for a variety of microorganisms. In several cases peptides were nutritionally superior to amino acids because the amino acid in peptide form was protected from degradative enzymes. As synthetic peptides have become more readily available the scope of peptide transport research has increased, especially over the last 10-15 years. Because of the large volume of work published in this area it is impractical to attempt a comprehensive survey. It is therefore proposed to limit this review to summarising the characteristics of peptide transport, providing only the more recent references. Further details of the earlier work can be obtained from one of the many reviews which have been published in this area (Sussman and Gilvarg, 1971; Barak and Gilvarg, 1975; Matthews

and Payne, 1975a, 1980; Payne, 1972,1975,1976,1977,1980; Payne and Gilvarg, 1971, 1978; Becker and Naider, 1980; Wolfinbarger, 1980).

Mechanisms for peptide uptake

There are several ways in which a peptide could be taken up to provide an amino acid source for a bacterial cell. 1) Peptide could be cleaved extracellularly and the amino acid residues transported into the cell. 2) Peptide could be taken up intact and then cleaved intracellularly to release the amino acid residues. 3) Peptide could be cleaved during translocation across the membrane, with release of the amino acid residues to the inside of the cell. These different mechanisms are depicted in Figure 1.3.

Mechanism 1) occurs in those organisms where there is extracellular peptidase activity. There have been many reports of organisms which produce extracellular proteases, however there are only a handful in which secreted enzymes with activity towards small peptides have been found. Those organisms which have been reported to possess extracellular peptidase activity include, Aeromonas proteolytica (Wilkes et al., 1973; Prescott and Wilkes, 1976), Bacillus cereus (Twardowski et al., 1977), B. licheniformis (Hall et al., 1966), B. subtilis (Ray and Wagner, 1972; Wagner et al.,

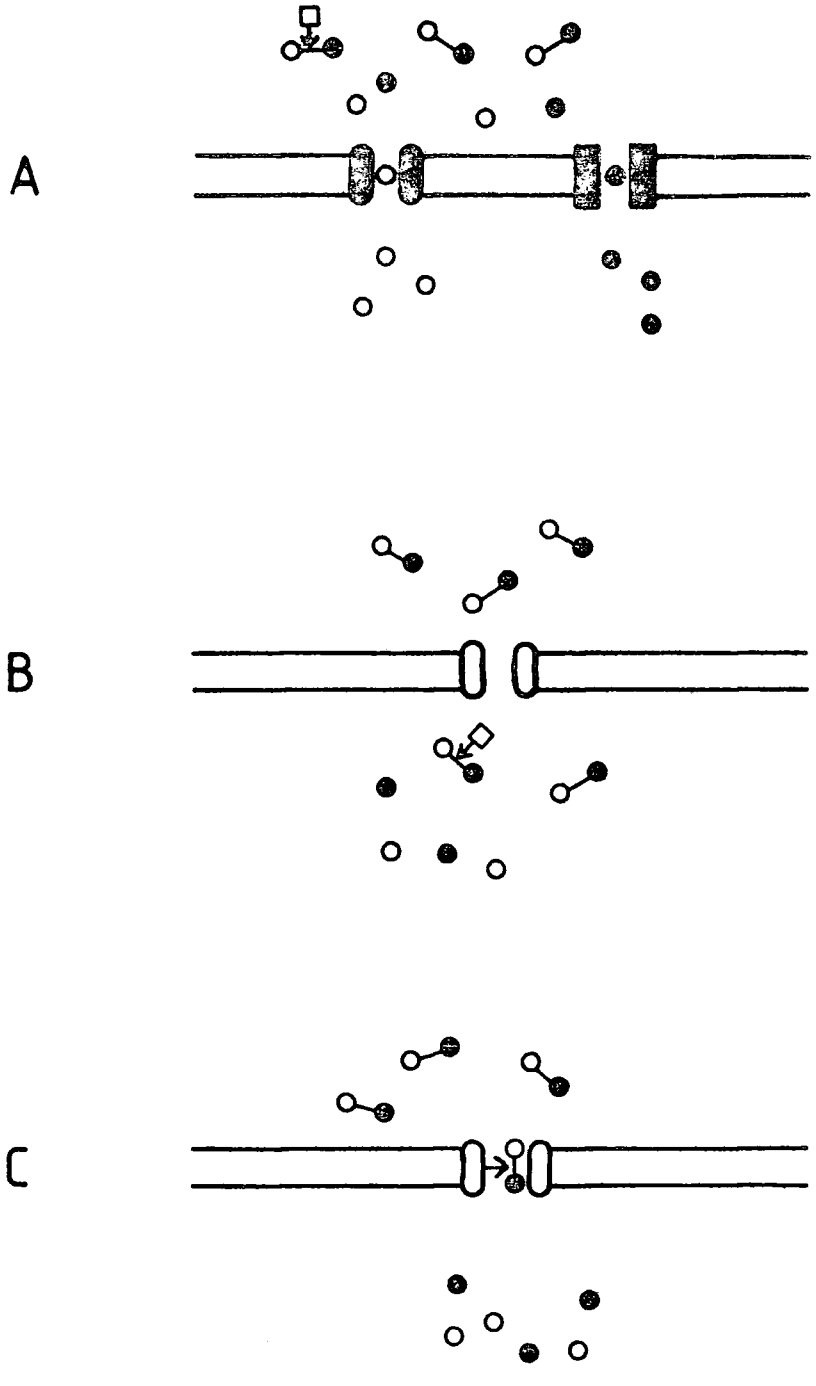


Figure 1.3 Mechanisms of peptide transport.

A, external peptide cleavage, followed by amino acid uptake; B, intact peptide uptake followed by intracellular cleavage; C, peptide cleavage as an integral part of uptake. , peptidase; , peptide; , amino acid; , cell membrane with permease.

1972), Clostridium histolyticum (Kessler and Yaron, 1976a,b), Saccharomyces cerevisiae (Frey and Rohm, 1979), Streptococcus cremoris and S. lactis (Law, 1977). Most bacterial cells produce peptidases involved in cell wall biosynthesis, however these usually have very restricted specificities and are not considered to play a general role in peptide utilisation.

In those organisms which do not display extracellular peptidase activity either mechanism 2) or 3) may be operating. In either case the following should be observed, a) lack of competition for uptake between free amino acid and peptide-derived amino acid, in contrast to mechanism 1) which is characterised by competition between free amino acid and amino acid released from peptide; b) uptake of peptides in amino acid permease-deficient mutants and vice versa; c) impermeant amino acid residues may be transported into the cell in peptide form.

A lack of competition can be demonstrated directly either by measuring uptake in the presence and absence of competitor or indirectly by isolating mutants defective in either peptide transport or amino acid transport and showing that amino acids or peptides respectively are still taken up. Studies in which amino acids have been shown not to compete with peptide transport have been reported for many species

including, E. coli (Cowell, 1974, Payne and Bell, 1977a), Bacteroides ruminicola (Pittman et al., 1967), Candida albicans, (Logan et al., 1979), Lactobacillus casei (Leach and Snell, 1960), Leuconostoc mesenteroides (Mayshak et al., 1966; Yoder et al., 1965), Neurospora crassa (Wolfinbarger and Marzluf, 1975), Saccharomyces cerevisiae (Becker and Naider, 1977), Streptococcus cremoris, S. lactis (Law, 1978), S. faecalis (Brock and Wooley, 1964; Nisbet, 1980) and Staphylococcus aureus (Perry and Abraham, 1979).

Studies using transport defective mutants have also been reported. Isolating mutants that cannot utilise peptides but can utilise amino acids does not directly show that the two transport systems are distinct, as peptidase deficiency could also prevent peptide utilisation, although this alternative is readily tested for. More commonly mutants resistant to toxic peptides have been isolated and these are almost invariably found to be peptide transport defective (see later in this section). Amino acid transport defective mutants that can still utilise peptides provide an alternative approach to studies on the separation of the systems. Such mutants have been reported for E. coli (De Felice et al., 1973; Guardiola and Iaccarino, 1971), Lactobacillus casei (Peters et al., 1953), Saccharomyces cerevisiae (Nisbet, 1980) and Salmonella typhimurium (Kustu and Ames, 1973).

Peptide transport has been shown, by the studies mentioned above, to be independent of extracellular hydrolysis in a number of species. Peptide transport must therefore, be either by mechanism 2) or 3) in these species. To distinguish between transport mechanisms 2) and 3), uptake of intact peptide must be demonstrated. The levels of intracellular peptidase activity towards natural L-peptides are generally much higher than transport rates e.g. in E. coli (Payne, 1972) or Pseudomonas aeruginosa (Miller and Becker, 1978) making intracellular detection of intact peptide difficult. There are two ways which have been used to overcome this problem, either using peptides resistant to peptidase activity or using mutants defective in peptidase activity. Glycylsarcosine and triornithine are two examples of intact peptides that have been detected intracellularly after uptake into E. coli (Payne and Bell, 1979). Payne and Nisbet (1980b) have shown intact peptide uptake in peptidase-deficient strains of Salmonella typhimurium.

Although these results indicate that intact peptide uptake followed by cytoplasmic cleavage does occur in E. coli and Salmonella typhimurium, the possibility that in addition a mechanism of cleavage concomitant with uptake cannot be totally excluded. There is no evidence to date in support of combined cleavage and uptake occurring in microorganisms, although it has been proposed as a mechanism in the mammalian

gut (Matthews, 1975; Ugolev, et al., 1977). There have been several reports of membrane-bound peptidases which might be involved in this type of peptide transport e.g. peptidase N in E. coli (Lazdunski et al., 1975), Pseudomonas aeruginosa (Miller and Becker, 1978) and Streptococcus cremoris and lactis (Law, 1977,1978). It is possible for such an enzyme, if it spanned the membrane, to bind peptides at the outer surface, cleave them as part of the translocation step and release the amino acid residues inside the cell. Peptidase N, which was first found associated with the cytoplasmic membrane of E. coli K-10 (Lazdunski et al, 1975) has been found to be located intracellularly in other strains of E. coli (Murgier et al., 1977). Peptidase N may be atypically located in E. coli K-10. An intracellular location for this enzyme would preclude its involvement in this mechanism of peptide transport. In addition, a mutant of E. coli lacking peptidase N activity did not show any reduction in peptide transport activity (Payne and Bell, 1979). It seems unlikely that a membrane-bound peptidase is active in peptide transport in E. coli, however at the moment it is best to keep an open mind in general for there may be such a mechanism occurring in the other species mentioned.

Peptide uptake and amino acid exodus

When the dansyl chloride assay was developed (Payne and Bell, 1979), which enables both amino acids and peptides in the medium to be visualised (see section 1.4), it became apparent that as peptide uptake proceeded there was also a rapid exodus of amino acid. This phenomenon has also been observed in a variety of other organisms, e.g. Streptococcus faecalis (Nisbet and Payne, 1980), Salmonella typhimurium (J.W. Payne, personal communication) and Staphylococcus aureus (Perry and Abraham, 1979). The amino acid exodus is presumably an attempt by the cell to regulate the levels of the amino acid pool so as to prevent any one amino acid from reaching levels which might be detrimental to cell metabolism.

The amino acids exported from the cell are usually the hydrolytically released peptide constituents or metabolically related residues. Certain amino acids such as Ala or Gly are metabolised little after hydrolysis and consequently almost all residues of these amino acids are exported during peptide uptake. Other amino acids such as Glu and Asp are rapidly metabolised after hydrolysis so that little of these amino acids is seen in the medium during peptide uptake. In these cases much of the amino acid may be deaminated, preventing its detection in the dansyl chloride assay, whether these products undergo exodus is not known. Some amino acids are metabolised to other amino acids which are then exported and

can be detected with the dansyl chloride assay, for example, in E. coli uptake of valine-containing peptides leads to proline exodus in addition to valine exodus (Payne and Bell, 1977b).

E. coli cells probably do not encounter high levels of a single peptide in the gut, rather a balanced range of peptides would be taken up which would alleviate the need for large scale amino acid exodus. Amino acid exodus can also influence the rates of uptake determined by the various peptide transport assays. Not only do these effects vary from one assay to another but they can also vary from peptide to peptide. A more detailed explanation of the effects of amino acid exodus on each uptake assay is provided in section 1.4.

The number and nature of peptide permeases

Several organisms have been shown to possess more than one peptide permease by studies of competition between peptides and the isolation of transport-deficient mutants. It should be borne in mind when assessing many of the early competition studies, that relatively insensitive and indirect growth assays were used, which limits the conclusions which can be drawn from such studies. The importance of ensuring that the peptide concentrations used in competition studies are

sufficiently high to saturate the transport system must be stressed, as concentrations below saturation may well show little competition between peptides even when they share the same permease. In addition, the widely varying affinities of permeases for different peptides indicates the need for large competitor: substrate ratios to determine whether or not competition is occurring.

The isolation of peptide transport-deficient mutants is much better evidence for the existence of more than one peptide permease. In E. coli, Payne (1968) isolated mutants resistant to triornithine which had lost the ability to utilise many oligopeptides but not dipeptides, indicating that E. coli possesses separate di-, and oligopeptide permeases. Later studies (Barak and Gilvarg, 1975; Naider and Becker, 1975) indicated that there was a further oligopeptide permease(s) of limited specificity.

The results of competition in growth tests indicate that in E. coli , dipeptides compete for transport to a limited extent with oligopeptides but that oligopeptides do not appear to compete with dipeptides (Payne, 1968). These results indicate that dipeptides can also use both the dipeptide permease and the oligopeptide permease, but that oligopeptides can only use the oligopeptide permease. It should be emphasised however that these results were obtained

from relatively insensitive assays.

Mutants defective in oligopeptide uptake but not dipeptide uptake have also been isolated in Salmonella typhimurium (Ames et al., 1973; Jackson et al., 1976). Streptococcus faecalis has been shown to possess two peptide permeases, but these do not correspond to the di- and oligopeptide permeases of E. coli and Salmonella typhimurium (Nisbet and Payne, 1980). In contrast, Saccharomyces cerevisiae has only one peptide permease (Marder et al., 1978; Nisbet and Payne, 1979a,b). Candida albicans (Davies, 1980) and Staphylococcus aureus (Perry and Abraham, 1979) have also been shown to possess a peptide permease which can handle both di- and oligopeptides. Whether these are the only transport systems in these species is not known.

There are also less conclusive reports from competition studies, indicating that the following species possess separate di-, and oligopeptide permeases, Lactobacillus casei (Dunn et al., 1957), Leuconostoc mesenteroides (Shelton and Nutter, 1964), Streptococcus spp. (Law, 1978; Rice et al., 1978) and Pseudomonas putida (Cascieri and Mallette, 1976a).

Mutants defective in oligopeptide transport have been reported to occur at a rate of approximately 1 in 10^5 cells in E. coli (Barak and Gilvarg, 1974) whereas a more usual

rate of occurrence for this type of spontaneous mutation is about 1 in 10^7 cells. Whether this high rate of mutation to *opp* is a general feature of oligopeptide type transport systems in other organisms, or is restricted to *E. coli* is not known. The frequency of loss of the other peptide transport systems has not been studied but would obviously have important implications for the rational design of peptide antibiotics.

Substrate specificities of peptide transport

The sheer number of possible combinations of natural L-amino acids in say a tetrapeptide (160,000) illustrates why peptide permeases must be able to recognise a wide range of amino acid residues. Although the amino acid constituents of peptides change, there are other features of the molecules which are consistently present, for example the peptide bond, the N-terminal amino group and the C-terminal carboxyl group. *A priori*, therefore, it would seem likely that these features would be the ones which a peptide permease recognises.

N-terminal α-amino group

In most of the microorganisms studied there is a requirement for a positively charged group at the N-terminus. In *E. coli*, the α-amino group can be substituted without

losing all transport activity, provided that a positive charge is retained e.g. N-monoalkyl derivatives. A substitution which removes the positive charge e.g. N-acyl derivatives, generally prevents uptake (Payne, 1971,1974). Similar conclusions have also been drawn for Neurospora crassa (Wolfinbarger and Marzluf, 1975), Pseudomonas putida (Cascieri and Mallette, 1976a), Salmonella typhimurium (Jackson et al., 1976), Staphylococcus aureus (Perry, 1981) and Streptococcus spp. (Law, 1977, 1978).

In Streptococcus faecalis (Shankman et al., 1960), Candida albicans (Lichliter et al., 1976) and Saccharomyces cerevisiae (Becker and Naider, 1977), there is some evidence that N-terminal substituted peptides without the positive charge can be utilised, although a more recent study in Candida albicans (Davies, 1980) could not detect any uptake of acetylated peptides. It should be noted that none of these studies has used direct sensitive transport assays, therefore it is difficult to draw firm conclusions from these results.

C-terminal α-carboxyl group

Peptide permeases do not generally have a strict requirement for an unmodified C-terminus, although modification invariably reduces the affinity of the permease

for such a peptide. Utilisation of C-terminal substituted peptides has been shown in Candida albicans (Davies, 1980), E. coli (Payne, 1973), Pseudomonas putida (Cascieri and Mallette, 1976a), Salmonella typhimurium (Ames et al., 1973) and Saccharomyces cerevisiae (Naider et al., 1974; Marder et al., 1977). Early studies with E. coli suggested that the dipeptide permease had a more strict requirement for an unsubstituted C-terminus than the oligopeptide permease (Payne and Gilvarg, 1968), however the dipeptide alafosfalin, which has a phosphono group at the C-terminus, has recently been shown to be transported via the dipeptide permease (Ringrose and Lloyd, 1979). Again, the insensitivity of the assays restrict the conclusions which can be drawn from these studies.

The α-peptide bond

Growth studies have indicated that an α-peptide bond is required by all peptide uptake systems studied to date. Peptides containing B, γ or ϵ linkages did not compete with α-linked peptides in E. coli (Payne, 1972). βAla-His was however utilised (Payne, 1973; Kirsh et al., 1978). Whether this uptake occurs via one of the peptide permeases is not known. β-linked peptides did not compete with α-linked peptides in Candida albicans (Davies, 1980), Pseudomonas putida (Cascieri and Mallette, 1976a), Streptococcus lactis

and Streptococcus cremoris (Law, 1978) or Salmonella typhimurium (Yang et al., 1977). In Candida albicans however γGlu-Ala and γGlu-Gly-Gly did show some limited ability to compete for peptide uptake.

Peptides with certain bond modifications are still transported, albeit less efficiently, by several species. Peptides in which the peptide bond nitrogen is methylated (e.g. glycylsarcosine) are still transported by E. coli (Payne and Bell, 1979), Saccharomyces cerevisiae (Nisbet and Payne, 1979a) and Streptococcus faecalis (Nisbet, 1980). These methylated bonds are resistant to peptidase action making them suitable for the demonstration of intact peptide uptake. Peptides in which an additional oxygen is inserted to the C-terminal side of the peptide bond nitrogen to give an aminoxy bond (see Appendix B for structure) are also transported via both the dipeptide and oligopeptide permeases in E. coli (Morley et al., 1983).

Side chain specificity

Competition for uptake has been shown between dipeptides and between oligopeptides in all the organisms tested to date (for reviews see Payne and Gilvarg, 1978; Payne, 1980), indicating that peptide transport systems can handle peptides containing a wide range of side chains. The only peptide

permeases reported to have a restricted specificity are the second oligopeptide permease of E. coli and the anionic peptide permease in Streptococcus faecalis (Payne et al., 1982). Growth studies indicated that the former system could only handle Thr, Leu and Met-containing tripeptides (Barak and Gilvarg, 1975; Naider and Becker, 1975). The growth assay used however, was most probably not sensitive enough to detect low level uptake of other peptides (see section 1.4). The anionic permease of Streptococcus faecalis has been shown by direct transport assays to be active only towards peptides with N-terminal Glu or Asp residues. The significance of this finding is not yet clear.

Stereospecificity of peptide uptake

All the peptide transport systems studied to date show a marked preference for L-amino acid residues. Intracellular peptidases lack significant activity towards D-amino acid residues making auxotrophic growth tests on peptides containing such residues difficult to interpret. Very few systematic studies have been reported, but for E. coli, the presence of a D-amino acid in the C-terminal position of a tripeptide is tolerated (Payne, 1980), but uptake is abolished when a D-amino acid is in the N-terminal position or the middle position. Only L-L dipeptides are transported in E. coli (Payne, 1980).

There seems to be some variation between strains of Saccharomyces cerevisiae in that Nisbet (1980) reported that any D-residue abolished uptake, whereas Naider and Becker (1975) reported that both L-Met-L-Met-D-Met and L-Met-D-Met-L-Met inhibited uptake of Met-Met-Met. Further studies are needed to clarify this point.

Size limit for peptide transport

Any size limit for uptake could be either a function of the peptide permease itself, or it could be a function of the permeability barrier in the overlying cell wall. Payne and Gilvarg (1968) showed that the upper size limit for peptide transport in E. coli occurred at a specific Stokes radius corresponding to a molecular weight of about 650, rather than a particular number of amino acid residues. Payne and Gilvarg suggested that this was probably due to an exclusion point in the cell wall rather than at the permease. Later work on the permeability of saccharides and polyethyleneglycols (Nakae and Nikaido, 1975; Decad and Nikaido, 1976) has shown that these molecules also have an exclusion limit at around 600 daltons. The porin pores in the outer membrane have now been established as the site of the exclusion limit in the outer membrane (see section 1.2).

In Neurospora crassa however, the size limit for peptide uptake appears to be a function of the permease (Wolfinbarger and Marzluf, 1975). Other studies have shown the pores in the cell wall to be permeable to molecules up to 4,750 daltons molecular mass (Trevithick and Metzenberg, 1966a,b) whereas the largest peptide transported was only a pentapeptide (approximately 500 daltons).

There have also been reports of a size limit for peptide transport in Pseudomonas putida (Cascieri and Mallette, 1976a), Saccharomyces cerevisiae (Naider et al., 1974; Marder et al., 1977) and Streptococcus lactis (Rice et al., 1978) but these do not indicate whether the size limit is caused by the permease or the cell wall.

The energetics of peptide transport

In principle, peptide transport could be a passive process, relying on intracellular hydrolysis to maintain a downward concentration gradient into the cell. Thus to demonstrate active transport it is necessary to show intact accumulation of peptide against a concentration gradient. In eukaryotic organisms there is the additional complication whereby peptide might be actively accumulated inside an organelle but only passively transported across the cell membrane. In this case, even accumulation of intact peptide

may not be evidence for active transport across the cell membrane.

There have been many studies in which metabolic poisons e.g. dinitrophenol have been shown to inhibit uptake. This provides indirect evidence for active peptide uptake in Bacteroides ruminicola (Pittmann et al., 1967), Candida albicans (Logan et al., 1979), E. coli (Cowell, 1974; Payne and Bell, 1979), Lactobacillus casei (Leach and Snell, 1959,1960), Leuconostoc mesenteroides (Yoder et al., 1965, Mayshak et al., 1966), Neurospora crassa (Wolfinbarger and Marzluf, 1975), Pseudomonas putida (Cascieri and Mallette, 1976a), Saccharomyces cerevisiae (Becker and Naider, 1977; Nisbet, 1980), Streptococcus faecalis (Nisbet, 1980) and Streptococcus lactis (Rice et al., 1978).

Intact accumulation of peptidase-resistant peptides has been demonstrated in E. coli (Payne, 1972; Payne and Bell, 1977b, 1979), Saccharomyces cerevisiae (Nisbet, 1980) and Streptococcus faecalis (Nisbet, 1980) while intact accumulation of peptides has been shown in peptidase-deficient mutants of E. coli (Kessel and Lubin, 1963) and Salmonella typhimurium (Jackson et al., 1976; Yang et al., 1977; J.W. Payne, personal communication).

There have been few reports of investigations of the energy coupling of peptide transport. In E. coli Cowell (1974) and Payne and Bell (1979) have shown that both di- and oligopeptide transport are linked directly to phosphate bond energy rather than the proton motive force. In contrast, Nisbet (1980) concluded that both Saccharomyces cerevisiae and Streptococcus faecalis were primarily linked to the proton motive force although some direct coupling to phosphate energy could not be excluded.

Peptide transport regulation

There has been very little work on peptide transport regulation in bacteria, but in those species studied, peptide transport appears to be constitutive with indirect regulation occurring via exodus of amino acids in some cases. Payne and Bell (1977b) reported that peptide uptake was reduced in the presence of several amino acids, however the mechanism of this effect is not clear. In Saccharomyces cerevisiae peptide transport has been shown to be repressed, along with several amino acid permeases, by the presence of ammonium ions (Becker and Naider, 1977; Nisbet and Payne, 1979a,b).

Peptide mimetic antibiotics

Peptide permeases are unusual among transport systems in the wide range of substrates which they are able to handle. The lack of specificity towards side chains and the C-terminus allows modified peptides containing otherwise impermeant amino acid derivatives to be transported into the cell (Ames et al., 1973; Fickel and Gilvarg, 1973) where peptidase action can release the toxic "warhead". The use of a permease in this way can enable low external levels of antibiotic to be toxic because the transport system may actively concentrate the toxic moiety inside the cell up to 1,000 fold.

To be useful as an antibiotic in chemotherapy, a peptide mimetic agent ideally should have the following features: a) It must be resistant to enzymic degradation on its way to the target cell; b) It must be rapidly transported into the target cell and transported slowly, if at all, by the host tissue; c) Once inside the target cell it must either be toxic intact or subject to rapid hydrolysis to release the toxic moiety; d) In the last instance, the "warhead" should not be transported by the target cell transport systems so that once it is released inside the cell it will not be able to get out, enabling very high intracellular concentrations to be attained.

Clearly the ability to rationally design a novel antibiotic relies on the availablility of detailed information about the substrate specificities of the peptide transport systems of the target organism and of the host. In addition, the specificities of the host peptidases must also be well characterised. A comprehensive survey of peptides with antimicrobial activity has been given by Ringrose (1980).

In the present work, several toxic peptide mimetics have been used to isolate resistant mutants (for structures of these, see Appendix B). Most mutants resistant to toxic peptides appear to be peptide transport deficient, even though resistance could also occur via a change in peptidase activity (a reduction for those peptides which must be cleaved to release the toxic moiety e.g. Ala-AlaP, or an increase for those peptides which are toxic intact e.g. triornithine), or a change in the target site. Presumably most peptides can be cleaved by several peptidases but are largely transported via one or two permeases, thus increasing the likelihood of resistant mutants being transport deficient. A mutant of Streptococcus faecalis resistant to Ala-AlaP (see Appendix B for structure) has been shown to be peptidase deficient (Payne et al., 1982), although other resistant mutants were transport deficient. This indicates that in S. faecalis Ala-AlaP is cleaved predominantly by one

peptidase enabling loss of that peptidase to prevent release of the toxic moiety. The infrequency of altered target sites probably indicates that either the peptide mimetic has several sites of action, or that cells containing altered target sites are rare or of low viability and are therefore infrequently detected.

1.4 METHODS FOR STUDYING PEPTIDE TRANSPORT

Introduction

Most studies of active transport involve the use of radioactively-labelled substrates and uptake is monitored by the accumulation of radioactivity in the cells or tissue being used. Unfortunately, very few radioactively-labelled dipeptides and no oligopeptides have ever been commercially available, therefore investigators must either synthesise labelled peptides themselves, in which case they are forced to draw conclusions from a very limited substrate range or other indirect methods of monitoring transport must be used. Unfortunately these indirect methods are relatively insensitive, and the fact that they are indirect limits the conclusions which may be drawn from them. Fortunately in the last five years, two new fluorescence-labelling assays have

been developed which have the advantages of being direct and sensitive and also allow the use of widely available non radioactively-labelled peptide substrates. The advantages and disadvantages of the direct fluorescence and radioactively-labelled peptide assays, which have been used in this study, will be discussed here. In addition it is pertinent to summarise the characteristics of the indirect assays to enable the reader to interpret better the results of earlier work.

Indirect methods

Growth assays

The majority of studies to date have used, in part at least, the growth of microorganisms as a measure of peptide uptake, using peptides as sources of amino acid (for reviews see Payne, 1976, 1977). The procedure normally followed is to monitor the growth of an amino acid auxotroph, by measuring the increase in turbidity, in a medium containing a peptide as the sole source of the required amino acid. It is important to determine that the strain possesses adequate intracellular peptidase activity towards the peptide tested but that it does not produce any extracellular peptidase activity. A wide range of peptides can be screened by this means although this procedure is slow and requires relatively

large amounts of peptide, and various auxotrophic strains.

The growth rate will depend on the rate of supply of amino acid although typically for rapidly transported peptides other factors will be growth limiting. Misleading results may arise when growth rates on different peptides are compared. For example, if a modification to a peptide reduces its uptake rate by 80% but transport rate of the unmodified form is ten times faster than required, then the modification will have no measurable effect. If, however, the same modification decreases the rate to the same extent in a peptide transported only marginally more quickly than required, then there may be a marked decrease in growth rate. In some species, the peptide may be rapidly transported, hydrolysed and the liberated amino acid residues undergo exodus. When all the peptide has been transported the rate of growth will be dependent on the reabsorption of the constituent amino acids via amino acid permeases. Under these circumstances growth will eventually be a measure of amino acid transport rather than peptide transport.

The assay can only be performed under conditions that allow growth, precluding experiments using transport inhibitors etc.. Competition studies can be performed using this assay and have provided much information on the specificities of the transport systems. Studies measuring

the inhibition of growth by toxic peptides and the cross resistance of transport-deficient mutants can also provide information about transport specificities. The main advantage of the auxotrophic growth assay is the wide range of substrates that can be used, and its disadvantage is the imprecise and limited conclusions which can be drawn from the results.

Monitoring enzyme synthesis

This method also monitors the utilisation of amino acid in peptide form but measures production of a single enzyme instead of overall growth (Bell et al., 1977). To start the assay, amino acid auxotrophic cells are resuspended in a medium which derepresses or induces production of an enzyme e.g. β-galactosidase. Peptide is added and the amount of enzyme synthesis is monitored. The enzyme production is proportional to the rate and and amount of peptide-bound amino acid supplied. This method is considerably more sensitive than the growth assays, requires less peptide and is faster. It is still however, an indirect assay and suffers from many of the disadvantages already discussed for growth assays.

Incorporation of radioactively-labelled amino acid

This method requires a strain auxotrophic for two amino acids. One of the required amino acids is supplied radioactively-labelled in excess of nutritional requirements, and the other is supplied in peptide form (Payne and Bell, 1977c). Protein synthesis can only take place in the presence of both amino acids so that, assuming excess radioactively-labelled amino acid, the rate of protein synthesis is proportional to the supply of amino acid in peptide form. Like the previous method, this assay is faster than the growth tests, requires less peptide and is more sensitive. It suffers from the disadvantages of being an indirect assay.

The last two assays have been used little as they were superceded by the direct fluorescence assays developed shortly afterwards.

Direct methods

Uptake of radioactively-labelled peptides

This method normally involves incubating a microbial suspension with a radioactively-labelled peptide, removing samples periodically, filtering off and washing the microorganisms followed by measuring the radioactivity retained in the microbial cells on the filter. Studies using

this method have been described for many species by many investigators e.g., in Candida albicans (Davies, 1980), E. coli (De Felice et al., 1973; Cowell, 1974; Neuhaus et al., 1977; Staros and Knowles, 1978; Payne and Nisbet, 1980b), Pseudomonas putida (Cascieri and Mallette, 1976a), Saccharomyces cerevisiae (Nisbet, 1980), Salmonella typhimurium (Jackson et al., 1976; Yang et al., 1977; Nisbet, 1980), Staphylococcus aureus (Perry and Abraham, 1979), Streptococcus faecalis (Nisbet, 1980), Streptococcus spp. (Law, 1978; Rice et al., 1978). The method is very sensitive, direct and fast. It does not require the use of amino acid auxotrophs or conditions which permit growth and transport kinetics can be measured. Unfortunately the range of available substrates is very limited, although this can be overcome by using competition studies, at the cost of losing the directness and some of the sensitivity of the assay.

In recent years, several problems associated with this method have been highlighted. Since the development of the dansyl chloride fluorescence assay it has become clear that in many organisms, amino acid exodus occurs concomitant with peptide transport. The radioactively-labelled peptide assay does not distinguish between a labelled peptide and a labelled amino acid, therefore exodus of labelled amino acid will produce a lowering of the apparent rate of transport. Although rapid sampling can partly overcome this problem,

there is evidence (J.W. Payne, personal communication), that in organisms such as *E. coli*, amino acid exodus commences within a few seconds of addition of peptide. Additionally, the transport rate observed can depend on which of the amino acid residues contains the radioactive label (Payne and Nisbet, 1980b). This is because some amino acids undergo more rapid exodus than others and thus loss of label is different depending on which amino acid is labelled. Loss of label has also been shown to occur via metabolic loss of CO_2 from [1-^{14}C]Gly-Phe and Gly-[U-^{14}C]Phe in *Staphylococcus aureus* (Perry and Abraham, 1979). A similar effect has also been observed in *E. coli*, *Salmonella typhimurium* and *Saccharomyces cerevisiae* using Ala-[U-^{14}C]Ala, Ala-Ala-[U-^{14}C]Ala, [1-^{14}C]Gly-Phe and Gly-[U-^{14}C]Phe (Nisbet, 1980).

The loss of counts through amino acid exodus and metabolism, when combined, can produce a large underestimate of the true rate of transport. Uptake rates and kinetic parameters obtained with this assay therefore may not accurately represent the true characteristics of peptide uptake.

Dansyl chloride assay

Dansyl chloride (1-dimethylamino naphthalene 5-sulphonylchloride) reacts with primary and secondary amines to form fluorescent derivatives. The amino groups of peptides and amino acids both react with dansyl chloride and the derivatives can be separated by two dimensional thin layer chromatography. In this way, amounts of peptide and amino acid less than 1nmol can be detected. Because fluorescent-labelled peptides are unlikely to be substrates for transport the peptides are labelled after incubation. The assay involves incubating cells with peptide, periodically removing samples and separating the cells and medium by filtration. Cell extracts can be prepared by boiling the cells in water. Samples of medium or cell extract are reacted with dansyl chloride and spotted on polyamide thin layer chromatography sheets. The dansyl derivatives are then chromatographed in two dimensions using three solvents. The spots are visualised under long-wave UV light and are identified by comparison with the positions of standards. Spot intensities can be quantified either by visual estimation using standard spots for comparison or using a thin layer scanner attached to a recording fluorescence spectrophotometer. Transport rates can thus be estimated from the loss of peptide from the medium.

Although this procedure is probably the least sensitive of the direct assays, it is still much more sensitive than any

of the indirect methods. The great advantage of using this method is that it allows changes in the concentrations of several peptide and amino acids to be monitored simultaneously. Dansylation of both the medium and cell extracts allows the fate of absorbed peptide to be followed, as shown by the studies in which amino acid exodus was demonstrated in E. coli (Payne and Bell, 1977b, 1979). The technique has also been used with yeast (Nisbet and Payne 1979a,b) and plants (Higgins and Payne, 1977). This assay has been described in more detail by Higgins (1979) and by Nisbet (1980).

Manual fluorescamine assay

Fluorescamine reacts with non-protonated primary amines to form intensely fluorescent derivatives (Udenfriend et al., 1972; De Bernardo et al., 1974). As the terminal amino groups of amino acids and peptides have different pKa values, fluorescamine exhibits selective reactions with amino acids and peptides dependent on the reaction pH. At pH 9 the reaction is optimal for amino acids whereas the optimum reaction for peptides occurs at pH 7-8. At pH 7-8 there is still a significant reaction with amino acids, however at about pH 6.2 the yield with peptide is much greater than that with amino acid (the yield with amino acid is less than 5% of that with isomolar peptide), whilst still being sufficiently

high to permit sensitive detection of peptides (Perrett et al., 1975). This differential labelling of peptides at pH 6.2 is the basis for the peptide uptake assay (Nisbet and Payne, 1979a,b). By altering the reaction pH to 9 the assay can also be used to measure amino acid uptake. The assay and the reactions of fluorescamine are described in more detail by Higgins (1979) and Nisbet (1980).

Medium samples are removed and filtered, as in the dansyl chloride assay, and samples of filtrate are reacted with fluorescamine at pH 6.2. The samples and fluorescamine solution must be mixed rapidly as the main reaction occurs in milliseconds. To achieve this, the sample is vortexed during addition of fluorescamine. The fluorescence of each sample is read in a fluorescence spectrophotometer and by comparing the values obtained with those of known peptide standards, the rate of peptide uptake can be calculated.

The fluorescamine assay is quicker and more sensitive than the dansyl chloride assay but cannot monitor peptides and amino acids simultaneously. The effect of amino acid exodus on this assay is negligible except under conditions where almost all of the peptide initially present in the medium has been transported, hydrolysed and undergone exodus. These conditions rarely apply during routine measurement of transport rates.

When peptide uptake is measured by both the fluorescamine and radioactively-labelled peptide assays in organisms in which amino acid exodus occurs, the rates obtained with the fluorescamine assay are invariably higher than those obtained with the radiolabel assay because of the interference of amino acid exodus and metabolism with the radioactively labelled peptide assay.

The fluorescamine assay is ideal for measuring transport kinetics and, like the other direct assays, can be used with a range of medium conditions enabling energy coupling etc. to be studied.

Continuous fluorescamine assay

The development of the continuous fluorescamine assay was a logical step from the manual fluorescamine assay. In this assay, as the name suggests, cell suspension is continuously removed from the incubation, filtered, mixed with buffer and fluorescamine and passed through a flow cell in the fluorescence spectrophotometer. For a detailed description of the assay see Nisbet (1980) and Payne and Nisbet (1981). The data from the spectrophotometer were recorded on a chart recorder in early experiments, but are now recorded on a microcomputer which is interfaced with the spectrophotometer. The components of the system are shown in diagrammatic form

in Figure 1.4.

Each assay is begun by adding peptide to the microbial suspension and starting the data recording program. The microbial suspension is drawn continuously from the incubation vial by the peristaltic pump, and through the filter apparatus to remove the microorganisms. The filtrate is then mixed with buffer to bring the pH to the correct value for reaction of the peptide with fluorescamine in a second mixing chamber. The resulting solution is then passed round a delay coil to allow air bubbles, which are driven out of solution by the isopropanol in which the fluorescamine is dissolved, to aggregate and to allow the fluorescence to stabilise. The air bubbles are then removed from the solution via a debubbler junction and the solution is passed through the spectrofluorimeter flow cell and its fluorescence is monitored. The spectrofluorimeter measures the fluorescence every 0.08sec and averages 8 readings to produce each datum point every 0.64sec. The microcomputer prints out each datum point as it receives it and also records it on a floppy disc for retrieval later (see section 2.6). If required, the reaction mixture can be collected after passing through the fluorimeter and the peptides and amino acids it contains can be separated using thin layer chromatography as in the dansyl chloride assay.

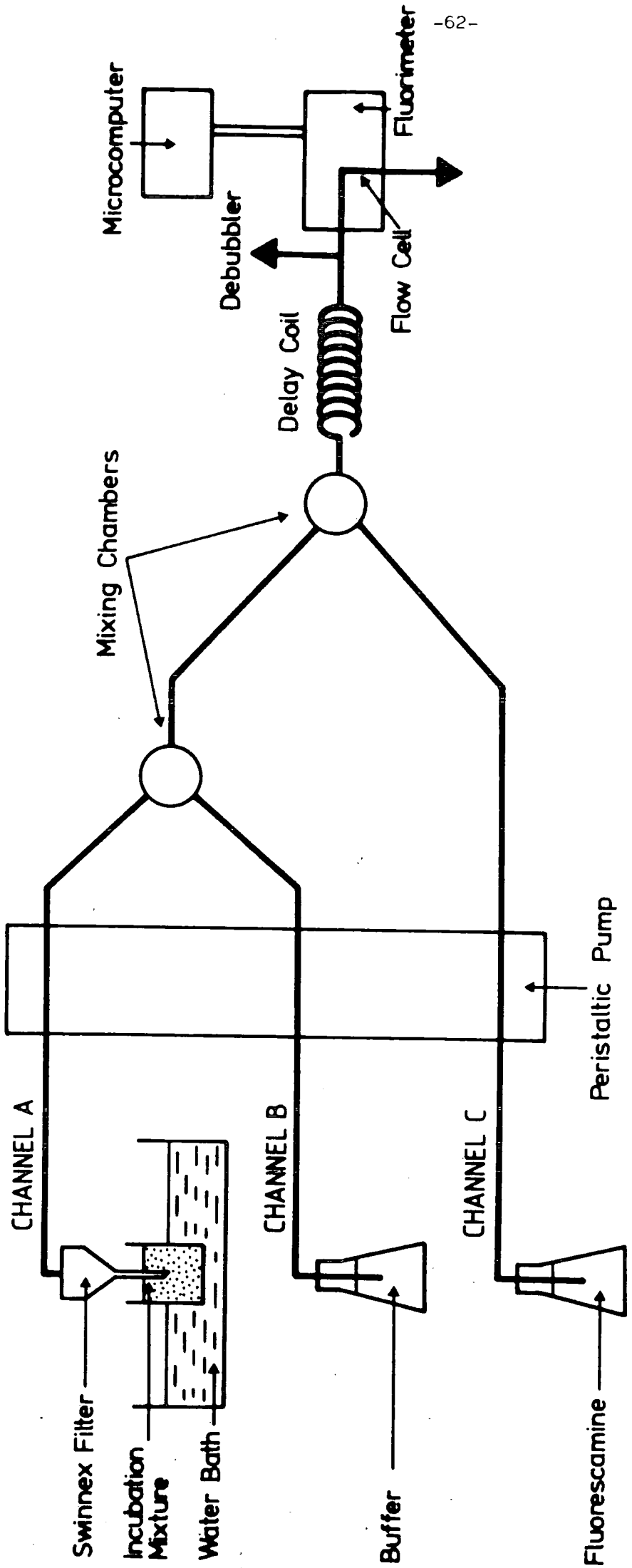


Figure 1.4 Schematic representation of the components of the continuous-flow fluorescamine assay.

There are considerable advantages in using the continuous fluorescamine assay rather than the manual fluorescamine assay. 1) The continuous assay produces much more data, and therefore more reliable results, than the manual assay. 2) Consistent and efficient mixing of medium and fluorescamine is crucial to the reproducibility of results and in these respects the mixing in the continuous assay is superior to that in the manual assay. 3) Results are obtained during the assay making it the quickest of all the peptide uptake assays and intrinsically an interactive system. 4) The data, once recorded by the microcomputer, are available for further processing to provide information such as transport kinetics (see section 2.6 and Appendix A).

The automated assay also has the other advantages of the manual fluorescamine assay and can be adapted to measure amino acid transport by using a buffer in channel B which will produce a solution pH of 9. The method has been used to measure peptide and amino acid uptake in E. coli, Streptococcus faecalis (Nisbet, 1980; Payne and Nisbet, 1981) and Candida albicans (J.W. Payne, personal communication).

The only significant problem still remaining with the use of this system is the time delay after the start of each assay before uptake rates can be measured, this arises from the longitudinal mixing in the filter holder, mixing chambers

and tubing. This causes a delay of about 2min before useful uptake data are produced (see Payne and Nisbet, 1981 and Appendix A for typical uptake profiles). This difficulty has been minimised by reducing the internal volume of the filter holder and mixing chambers to the minimum necessary for free flow and by using tubing of a smaller internal diameter, and may possibly be improved still further by additional refinements to the physical construction of the system. Uptake rates in this initial period can be measured by relating the profile of an experimental curve to that of a control curve with no uptake, but this procedure can only be done by hand at present and is laborious. Work is in progress on this problem (J.W. Payne, personal communication), and in the near future a program should be completed that will enable the microcomputer to calculate the rates of uptake in this initial period.

1.5 NITROGEN METABOLISM IN PSEUDOMONAS AERUGINOSA

Introduction

The members of this family are varied and are found in a wide variety of environments (Palleroni, 1981). Pseudomonads have been isolated from soil, water, animal and plant tissues and even sources such as mineral oils and kerosene (Stanier *et al.*, 1966). Many members of the group are able to metabolise a large number of different organic compounds.

The most studied member of the Pseudomonaceae is *Pseudomonas aeruginosa*, the type species of the genus Pseudomonas. *Pseudomonas aeruginosa* is an opportunistic pathogen of man and has also been shown to be phytopathogenic (Palleroni, 1975). Although only weakly pathogenic, the high resistance of *P. aeruginosa* to many antibiotics and disinfectants, and its ability to survive and grow in moist environments with very low nutrient levels e.g. distilled water supplies, make it an important organism in hospital infections (Lowbury, 1975).

Most strains of the species can utilise over 80 organic compounds as sources of carbon including carbohydrates, alcohols, saturated and unsaturated fatty acids, amino acids, amines and amides but not single carbon compounds (Stanier *et*

al., 1966). A wide variety of substances can be used as nitrogen sources such as nitrate, ammonium, urea, amino acids, amines and amides (Stanier et al., 1966).

In this section the areas of the metabolism of Pseudomonas aeruginosa which are most relevant to the study presented here will be surveyed. These include nitrogen assimilation, the regulation of nitrogen metabolic pathways and active transport.

Nitrogen assimilation and its regulation

The major sources of nitrogen for Pseudomonads are nitrate, which is reduced via nitrite to ammonia (Painter, 1970), ammonia itself and amino acids. Ammonia, the preferred nitrogen source, is assimilated by two routes involving either glutamate dehydrogenase (GDH) under conditions of high ammonia concentration, or the combined action of glutamine synthetase (GS) and glutamate synthase (GOGAT; L-glutamine: 2-oxoglutarate aminotransferase) under conditions of low ammonia concentration (Brown et al., 1973). Amino acids can either be used directly in protein synthesis or can be used as both carbon and nitrogen sources for other biosynthetic pathways via glutamate and glutamine depending on the nutritional status of the organism (Tyler, 1978). Although the mechanisms of nitrogen control in P. aeruginosa

are not well understood, it is likely that the enzymes involved in glutamine and glutamate metabolism will be involved in any such control, as glutamine and glutamate are central to most pathways of nitrogen flow in the cell.

GDH catalyses the production of glutamate from ammonia and 2-oxoglutarate (Tyler, 1978). The regulation of GDH production varies from organism to organism, for example, in P. aeruginosa GDH production appears to be only derepressed under conditions of excess ammonia (Brown et al., 1973) whereas in S. typhimurium, ammonia concentration has no effect on GDH regulation (Brenchley et al., 1975). The sructural gene for GDH (gdhA) in S. typhimurium has recently been cloned so that the activity of the promoter and other regulatory regions can be investigated (Miller and Brenchley, 1984). This pathway of ammonia assimilation is energetically more efficient than the coupled GS-GOGAT pathway except at low ammonia concentrations (Tyler, 1978). Glutamate synthase (GOGAT) catalyses the transfer of the amide group from glutamine to 2-oxoglutarate to produce 2 molecules of glutamate (Tyler, 1978). GS, the third enzyme involved in glutamine/glutamate metabolism, can then combine the glutamate with ammonia at the expense of an ATP to reform glutamine. Because GS has a relatively high affinity for ammonia and the pathway is ATP-driven, ammonia can be more efficiently assimilated at low concentrations by the GS-GOGAT

pathway than the GDH pathway.

GS is the most studied of the enzymes involved in nitrogen assimilation as it has long been thought to be the major regulatory factor for these pathways in enteric bacteria (Magasanik 1982). The activity of GS is regulated by the interconversion of active and inactive forms by divalent cations, by cumulative feedback inhibition by end-products of glutamine metabolism and by the state of adenylation of the molecule (Tyler, 1978). Magasanik *et al.* (1974) proposed that GS controls the synthesis of several enzymes involved in nitrogen assimilation e.g. histidase, GDH, urease and GS itself, by acting as a positive transcriptional control factor. It was also proposed that the level of adenylation controlled the ability of GS to act as a positive control factor. Further evidence in *E. coli* indicates that there is another regulatory locus closely linked to but separate from the structural gene of GS (*glnA*) (Pahel and Tyler, 1979). the groups of Magasanik and Tyler have shown that this locus consists of two cistrons *glnL* and *glnG*, and that the products are involved in both positive and negative control of *glnA* (Chen *et al.*, 1982; Pahel *et al.*, 1982; MacNeil *et al.*, 1982). Furthermore *glnA*, *glnL* and *glnG* comprise a single operon with two promoters, one of which produces transcription of the whole operon and one which is sited downstream of *glnA* and produces transcription of *glnL* and

glnG when the production of glnA is repressed (Chen et al., 1982; Pahel et al., 1982; Gutterman et al., 1982). Hanau et al. (1983) have reported a similar position in S. typhimurium. The situation in P. aeruginosa is less clear.

Mutants lacking GS (GlnR) have been described (Janssen et al., 1981) and indicate a key role for glutamine or a glutamine product in nitrogen control. A partial revertant of a GlnR mutant was caused by a mutation located near the structural gene for GS, suggesting that there may be a regulatory gene analogous to the one which occurs in E. coli (Janssen et al., 1982).

The glutamine synthetase regulatory system is also involved in the control of nitrogen assimilation via several other routes, so that under nitrogen-limited growth, synthesis of several catabolic pathways is derepressed. The number of pathways controlled by GS varies between species with asparagine and tryptophan utilisation being controlled by GS in Klebsiella aerogenes but not in E. coli (Tyler, 1978). In P. aeruginosa catabolism of methionine, proline and tryptophan is controlled in this way (Janssen et al., 1982). Recently Bender et al. (1983) reported a new locus (nac) in Klebsiella aerogenes, which is involved in the regulation of catabolic pathways by ammonia concentration. Whether this locus also occurs in other organisms is not yet

known.

Amino acid metabolism

P. aeruginosa is capable of synthesising all of the protein amino acids and therefore does not require any of them to be present in the growth medium. The amino acid biosynthetic pathways in P. aeruginosa are similar to those established in other bacteria. The degradative pathways are more varied so that even some D-amino acids e.g. D-lysine can be utilised (Miller and Rodwell, 1971).

Differences between enteric bacteria and P. aeruginosa occur in the regulation of both enzyme activity and enzyme synthesis. At the transcriptional level, the structural genes of enzymes for a biosynthetic pathway are more widely scattered in P. aeruginosa than in E. coli, for example, histidine biosynthetic genes are found in five groups in P. aeruginosa whereas they form a single operon in E. coli (Mee and Lee, 1969). The control of transcription of the his biosynthetic genes in P. aeruginosa is complex with as many as five regulatory genes. The major control of metabolic flow along the metabolic pathways in Pseudomonads appears to be via feedback inhibition of enzyme activity (Clarke and Ornston, 1975a). Where a branched pathway requires feedback control by several products, the enteric bacteria produce

isofunctional enzymes, each controlled independently, so that even in the presence of an excess of one product the pathway can still function to produce the other products. In Pseudomonads however, single enzymes with more complex modes of inhibition are often present. For example, the first step in the pathway of aromatic amino acid biosynthesis is catalysed by three isoenzymes of DAHP synthetase in E. coli, each inhibited by a different amino acid, whereas in P. aeruginosa a single DAHP synthetase enzyme is inhibited by tyrosine, tryptophan and phenylpyruvate, with a combination of the three inhibitors producing a cumulative effect (Jänssen et al., 1973). The amino acid biosynthetic pathways of P. aeruginosa and their regulation are described in more detail by Clarke and Ornston, 1975b)

The amino acid catabolic pathways are organised and regulated in a different manner to the biosynthetic pathways in Pseudomonads. The genes coding for these catabolic pathways tend to be clustered, unlike the biosynthetic genes, and there is often more than one pathway present for the catabolism of a single compound even in a single strain (Clarke and Ornston, 1975b). Many catabolic pathway genes are carried on transmissible plasmids which is likely to allow a population to grow on a wide range of potential substrates. These plasmids may often be lost in laboratory strains so that such strains would not possess such a wide

substrate range as a wild population.

The genes coding for amino acid catabolism tend to be coordinately induced by the presence of the amino acid or one of the early intermediates of the pathway. These catabolic pathways are also controlled by catabolite repression. Under conditions of low cAMP, catabolic pathways are generally repressed. In *E. coli*, glucose and other rapidly utilised carbon sources can cause severe catabolite repression, however in *P. aeruginosa* glucose is a relatively poor substrate and it is the intermediates of the TCA cycle e.g. succinate which produce the strongest catabolite repression. End product inhibition and end product repression also occur and provide further levels of control over the activity of the catabolic pathways although the major control seems to be substrate induction. The supply of substrates for the catabolic pathways is controlled by the availability of nutrients and by the biosynthetic pathways (Clarke and Ornston, 1975b).

Regulation of active transport

Just as many of the metabolic pathways in Pseudomonas are inducible, so are many of the transport systems. Induction of the transport systems is inhibited by chloramphenicol, indicating that *de novo* protein synthesis occurs, and not

just an activation of already synthesised protein (Clarke and Ornston, 1975a). The inducible uptake systems include those for glucose, glycerol and mandelate (Midgley and Dawes, 1973; Tsai et al., 1971; Higgins and Mandelstam, 1972). These transport systems are usually induced by the presence of their transport substrate and the same mechanisms e.g. catabolite repression, which repress the metabolic pathways, also repress the respective transport systems.

Other transport systems, e.g. those for amino acids and peptides, have been shown to be constitutive (Kay and Gronlund, 1969a; Cascieri and Mallette, 1976a). As the amino acid catabolic pathways are inducible it might be expected that the permeases would also be inducible, however, the only inducible amino acid permease activity in P. aeruginosa reported to date is the 10-fold increase in proline permease activity induced in the presence of proline (Kay and Gronlund, 1969b). Kay and Gronlund (1969a) showed that the presence of the substrate either as the sole carbon or nitrogen source only caused a slight increase in the uptake of alanine, glutamate and glycine, while leucine and valine transport activity fell and the uptake of other amino acids was unaffected. The rates of transport of amino acids into P. aeruginosa range from 0.2nmol min^{-1} mg dry $weight^{-1}$ (cysteine) to 8nmol min^{-1} mg dry $weight^{-1}$ (leucine) (Kay and Gronlund, 1969a).

Peptide transport has been shown to be constitutive in both P. aeruginosa and P. putida (Miller and Becker, 1978; Cascieri and Mallette, 1976a) although the possibility of inducing higher levels of activity has been given little attention. Peptide uptake in Pseudomonas has been largely studied using growth tests, therefore there is relatively little information regarding uptake kinetics, however rates appear to be similar to those for amino acid uptake and approximately ten times slower than peptide transport in E. coli.

2 MATERIALS AND METHODS

2.1 MATERIALS AND INSTRUMENTATION

Materials

Phosphono peptide derivatives were gifts from Dr. W.J. Lloyd, Roche Products Ltd., Welwyn Garden City. Aminoxy peptide derivatives were gifts from Dr. J.W. Morley, I.C.I. Pharmaceuticals Ltd., Alderley Park. Radioactively-labelled Gly-[U ^{14}C]Phe was from the Radiochemical Centre, Amersham; radioactively-labelled Ala-Ala-[U ^{14}C]Ala was a gift from Dr. W.J. Lloyd, Roche Products Ltd. Welwyn Garden City. Peptides were either from Sigma (London) Ltd., Poole or Uniscience Ltd., Cambridge. Fluorescamine was either a gift from Dr. W.J. Lloyd, Roche Products Ltd., Welwyn Garden City or was obtained from Sigma (London) Ltd., Poole. Dansyl chloride was from British Drug Houses Ltd.. Agar, Tryptone and Yeast Extract were obtained from Difco Ltd. or Oxoid Ltd.. Syringes and petri dishes were from Sterilin Products Ltd.. All other chemicals used were obtained either from Sigma (London) Ltd. or from British Drug Houses Ltd. and were of Analar grade or the highest grade available.

Instrumentation

The fluorimeter used in this study was a Perkin Elmer 1000 with a flow cell accessory. The microcomputer used for logging and processing the data from the continuous flow peptide uptake assay was a 380Z from Research Machines (Cambridge Ltd.) with double mini (7in) floppy disc drive units linked to an Anadex 9001 dot matrix printer and a Sony video monitor. Maxell MD2 double sided, double density floppy discs with a capacity for 72K of random access memory were used. The 380Z was fitted with the high resolution graphics board , additional memory board and arithmetic chip. The data logging and processing programs were written by Dr J.T. Gleaves. The scintillation counter used in the radioactive label uptake studies was a Packard Prias Tri-Carb liquid scintillation counter. Centrifuges used were all MSE centrifuges, an HS18 for cell harvesting and other low to medium speed work and a Prepspin for the high speed spins required during cell membrane fraction preparations. The sonicator used to break open cells was an MSE Soniprep. A Grant water bath (type SS-30) was used for growing cells in liquid media.

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used are shown in Table 2.1. The media used for growth and storage of each strain are shown in Table 2.2.

Media

The following media were used:

Minimal Media

A+C (Davis and Mingioli, 1950)

7.0g K_2HPO_4

3.0g KH_2PO_4

0.1g $MgSO_4.7H_2O$

1.0g $(NH_4)_2SO_4$

1.0ml 10% w/v Sodium Citrate

Made up to 1 litre with distilled water.

M9 (Miller, 1972)

6.0g Na_2HPO_4

3.0g K_2HPO_4

0.5g NaCl

Table 2.1 Bacterial strains used

Strain	Lab No	Relevant Genotype	Source
M26-26	M26-26	lys	A
M26-26	PA0112	lys opp	A
M26-26	PA0107	lys dpp	A
M26-26	PA0119	lys dpp opp	A
M26-26	PA0113	lys opp dpp	A
M26-26	PA0122	lys dpp opp opt	A
KL723	4251	thr-1 leuB6 proA2 his-4 recA13 ArgE3 thi-1 ara-14 lacY1 galK2 xyl-7 mtl-1 rpsL31 tsx-33 λ^- supE44 (contains F′ F104)	B
KL719	4282	leuB6 proC32 purE42 trpE38 recA1 metE70 thi-1 ara-14 lacZ36 xyl-5 mtl-1 azi-6 rpsL109 tonA23 tsx-67 λ^- supE44 (contains F′ F254)	B
E5014	4288	DE5 relA1? thi-1 mal-24 rpsE2112 λ^- supE44 (contains F′ F128)	B
KL718	4287	pyrD34 trp-45 his-68 tyrA2 recA1 thi-1 galK35 xyl-7 mtl-2 malA1 rpsL118 λ^R λ^- (contains F′ F152)	B
KL731	4254	leuB6 recA1 hisG1 thyA23 argG6 metB1 tonA2 tsx-1 λ^R λ^- supE44 (contains F′ F116)	B
KL708	4248	leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 xyl-7 mtl-2 malA1 rpsL104 tonA2	B

		tsx-1 λ^R λ^- supE44 (contains F′ F141)	
MAF1	4289	leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R λ^- supE44 (contains F′ F140)	B
DFF1	4326	leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R λ^- supE44 (contains F′ F150)	B
KL704	4280	leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R λ^- supE44 (contains F′ F129)	B
KL709	4279	pyrD34 trp-45 his-68 tyrA2 recA1 thi-1 galK35 xyl-7 mtl-2 malA1 rpsL118 λ^R λ^- (contains F′ F142)	B
KL711	4291	pyrD34 trp-45 his-68 tyrA2 recA1 thyA33 thi-1 galK35 xyl-7 mtl-2 malA1 rpsL118 λ^R λ^- (contains F′ F143)	B
KL701	4256	pyrD34 trp-45 his-68 recA1 thi-1 galK35 xyl-7 mtl-2 malA1 rpsL118 λ^R λ^- (contains F′ F123)	B
KL703	4253	pyrD34 trp-45 his-68 recA1 thi-1 galK35 xyl-7 mtl-2 malA1 rpsL118 λ^R λ^- (contains F′ F126)	B
GMS724	5505	aroD6 recA1 metB1 lacY1 galK2 man-4 rpsL700 tsx-29? supE44? (contains F′ F500)	B
JE5519	5760	aroD argE lac gal man rpsL nalA recA1	B

		(contains F′ F506)	
KL729	4258	leuB6 hisG1 recA1 argG6 metB1 lacY1 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R gal-6 λ^- supE44 (contains F′ F111)	B
KL729	4260	leuB6 hisG1 recA1 argG6 metB1 lacY1 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R gal-6 λ^- supE44 (contains F′ F112)	B
KL706	4265	leuB6 hisG1 recA1 argG6 metB1 lacY1 xyl-7 mtl-2 malA1 rpsL104 tonA2 tsx-1 λ^R gal-6 λ^- supE44 (contains F′ F133)	B
KL732	4255	thr-1 leuB6 pro-27 hisG1 recA1 thyA25 thi-1 pyrB31 ara-13 lacY1 gal-6 xyl-7 malA1 rpsL9 tonA2 λ^R λ^- supE44? (contains F′ F117)	B
X573	6350	serA12 λ^- supE42 T3r (contains F′ F254)	B
P400	H1	thi thr leu argE proA str non mtl xyl ara galK lacY supE	C
P400:6	H2	thi thr leu argE proA str non mtl xyl ara galK lacY supE ompC	D
P400:6hIr	H3	thi thr leu argE proA str non mtl xyl ara galK lacY supE ompC ompA	E
P530	H4	thi thr leu argE proA str non mtl xyl ara galK lacY supE ompB	F
CM6	NC1	thyA drm tonA mal kmt-7	G
CM7	NC2	thyA drm tonA mal kmt-7 ompB	G

AB2847	NC3	aroB351 tsx-354 mal354 supE42? $\lambda^R \lambda^-$	G
T19	NC4	tsx-354 supE42? ompB	G
AB2847	B1	aroB malT thi tsx	H,I
P8	B2	aroB malT thi tsx tonA	I
BR158	B3	aroB malT thi tsx tonB	H,I
RK3931	B4	aroB malT thi tsx tonA	H,I
WA28	B5	aroB malT thi tsx fecB	H,I
WA380	B6	aroB malT thi tsx fecA	H,I
W3110	B7	Wild Type	J
KB419	B8	lamB	J
KB423	B9	tsx	J
JE5512	B10	HfrC man pps	K
JE5513	B11	HfrC man pps lpp	K
JF568	568	aroA357 ilv-277 metB65 his-53 pro purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63	L
JF694	694	aroA357 ilv-277 metB65 his-53 pro purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompC264 ompF254 nmpA1	L
JF699	699	aroA357 ilv-277 metB65 his-53 pro purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompA	L
JF700	700	aroA357 ilv-277 metB65 his-53 pro purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompF ompA	L
JF701	701	aroA357 ilv-277 metB65 his-53 pro	L

		purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompC264	
JF703	703	aroA357 ilv-277 metB65 his-53 pro purE41 cyc-1 xyl-14 lacY29 rpsL77 tsx63 ompF254	L
SH5014	NS1	rfaA66 thr his-6116 ilv	M
SH5551	NS2	rfaA66 thr his-6116 metA22 metE551 tryB2 strA120 H1-b H2-e,n,x xyl-404 ilv ompD106	M
SH6017	NS3	rfaA66 thr his-6116 metA22 metE551 tryB2 strA66 H1-b H2-e,n,x xyl-404 ilv ompC336	M
SH6260	NS4	rfaA66 thr his-6116 ilv ompC ompD	M
HN407	NS5	galE ompC ompD ompF+++	N
PAO1	P1	Wild Type	O
PA08	P2	met-28 ilv-202 str-1 $FP2^{-}$	O
6749	P3	Wild type	P

Strain AB2847 was obtained from both T. Nakae and V. Braun. Each strain was used as a wild type reference for the strains derived from it.

A full decription of each F′ is provided in table 3.n

All M26-26 strains are Escherichia coli W

All F′ kit strains are Escherichia coli K12

NC1 and NC2 are Escherichia coli B/r

NS1-NS5 are Salmonella typhimurium

P1-P3 are Pseudomonas aeruginosa

All other outer membrane protein-deficient strains are

Escherichia coli K12

Source references:

A. See section 3.2

B. F′ kit from B. Bachmann, CGSC, Yale University.

C. Skurry et. al. (1974)

D. Schmitges and Henning (1976)

E. Henning et. al. (1978)

F. Davies and Reeves (1975)

G. Von Meyenburg (1971)

H. Kadner et. al. (1980)

I. Braun et. al. (1976)

J. Braun and Krieger-Brauer (1977)

K. Nikaido et. al. (1977)

L. Nikaido et. al. (1983)

M. Nurminen et. al. (1976)

N. Nakae and Ishii (1978)

O. National Collection of Industrial Bacteria, Aberdeen

P. National Collection of Type Cultures, London

Table 2.2 Growth media used for each strain

Str No	Growth Medium	
	Minimal	Complex
~~PA01~~ M2626	A+C + lys glc	L-broth
PA0102	A+C + lys glc	L-broth
PA0107	A+C + lys glc	L-broth
PA0107 19	A+C + lys glc	L-broth
PA0102 13	A+C + lys glc	L-broth
PA0107 22	A+C + lys glc	L-broth
4248	A+C + his leu met glc	L-broth
4251	A+C + thi arg his glc	L-broth
4254	A+C + arg his leu met glc	L-broth
4255	A+C + thi his leu pro thr thy glc	L-broth
4256	A+C + thi his ura glc	L-broth
4258	A+C + arg his leu glc	L-broth
4260	A+C + arg his leu glc	L-broth
4265	A+C + arg his leu glc	L-broth
4279	A+C + thi his trp ura glc	L-broth
4280	A+C + leu arg met glc	L-broth
4282	A+C + thi met trp leu glc	L-broth
4287	A+C + thi ura trp his tyr gal	L-broth
4288	A+C + thi lac	L-broth
4289	A+C + leu his met glc	L-broth
4291	A+C + thi his trp ura glc	L-broth
4323	A+C + thi thr trp thy glc	L-broth
4326	A+C + leu met arg glc	L-broth

5212	A+C + his gal	L-broth
5215	A+C + trp lac	L-broth
5218	A+C + met lac	L-broth
5505	A+C + met man	L-broth
5104	A+C + thi his leu thr arg gal	L-broth
H1	A+C + thi thr leu arg pro glc	L-broth
H2	A+C + thi thr leu arg pro glc	L-broth
H3	A+C + thi thr leu arg pro glc	L-broth
H4	A+C + thi thr leu arg pro glc	L-broth
NC1	A+C + thi glc	L-broth
NC2	A+C + thi glc	L-broth
NC3	A+C + glc	L-broth
NC4	A+C + glc	L-broth
B1	M9 + tyr trp phe thi glc cit phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B2	M9 + tyr trp phe thi glc cit phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B3	M9 + tyr trp phe thi glc dhba phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B4	M9 + tyr trp phe thi glc cit phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B5	M9 + tyr trp phe thi glc cit phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B6	M9 + tyr trp phe thi glc cit phba paba $MgSO_4$ $CaCl_2$ $FeCl_3$	L-broth
B7	M63 + glc	L-broth

B8	M63 + glc	L-broth
B9	M63 + glc	L-broth
B10	M63 + $MgSO_4$ glc	L-broth +5mM $MgSO_4$
B11	M63 + $MgSO_4$ glc	L-broth +5mM $MgSO_4$
568	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
694	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
699	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
700	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
701	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
703	A+C + phe tyr trp ile val met his pro ade gua glc	L-broth
NS1	A+C + thr his ile val glc	L-broth
NS2	A+C + thr his met try ile val glc	L-broth
NS3	A+C + thr his met try ile val glc	L-broth
NS4	A+C + thr his ile val glc	L-broth
NS5	A+C + glc	L-broth
PA01	PMM or PTM or A+C + glc	L-broth or Luria
PA08	PMM or PTM or A+C + met ile val	L-broth

	glc	or Luria
6799 ~~A21~~	PMM or PTM or A+C + glc	L-broth
		or Luria

Abbreviations used:- ade, adenine; arg, arginine; cit, citrate; dhba, dihydroxybenzoic acid; gal, galactose; glc, glucose; gua, guanine; his, histidine; ile, isoleucine; lac, lactose; leu, leucine; lys, lysine; man, mannose; met, methionine; paba, p-aminobenzoic acid; phba, p-hydroxybenzoic acid; phe, phenylalanine; pro, proline; thi, thiamine; thr, threonine; thy, thymine; trp, tryptophan; tyr, tyrosine; ura, uracil; val, valine; All media prepared as described in the text. All supplements used at the concentrations given in the text.

PMM Pseudomonas Minimal Medium
PTM Pseudomonas Tris Minimal Medium
(see Text)

1.0g NH_4Cl

10ml 1mM $CaCl_2$ (added after autoclaving)

Made up to 1 litre with distilled water.

M63 (Miller, 1972)

13.6g KH_2PO_4

2.0g $(NH_4)_2SO_4$

0.5mg $FeSO_4.7H_2O$

Titrated to pH7.0 with KOH and made up to 1 litre with distilled water.

Pseudomonas Minimal Medium (Miller and Becker, 1978)

60mM Potassium Phosphate buffer pH7.2

1.7mM Sodium Citrate

0.4mM Magnesium Sulphate

7.5mM Ammonium Sulphate

Pseudomonas Tris Minimal Medium (Miller and Becker, 1978)

120mM Tris-HCl pH7.2

2.0mM KCl

1.6mM $MgSO_4$

9.0uM $FeCl_3$

8.0mM $(NH_4)_2 SO_4$

All minimal media were routinely prepared as 10x concentration stocks and added to sterile flasks containing water to dilute the media to the correct concentrations. All supplements were sterilised separately and added to the flasks after the addition of medium.

Supplements

All minimal media were supplemented with 0.5% D-Glucose except for some of the gene mapping F′ donor strains (see Table 2.2) which were grown in media containing either galactose (0.1%), lactose (0.5%) or mannose (0.3%). Amino acids were added to a final concentration of 50ug ml^{-1}, citrate to 1mM, dihydroxybenzoic acid, p-hydroxybenzoic acid and p-aminobenzoic acid to 40uM, thiamine to 5ug ml^{-1}, thymine to 4ug ml^{-1}, adenine to 50ug ml^{-1}, guanine to 50ug ml^{-1} and uracil to 25ug ml^{-1}.

Complex Media

L-broth (Bertani, 1950)

10g tryptone

5g yeast extract

5g NaCl

1g glucose

Made up to 1 litre with distilled water

Luria broth (Miller and Becker, 1978)

10g tryptone

5g yeast extract

10g NaCl

0.1g NaOH

Made up to 1 litre with distilled water

Glucose broth (Miller, 1972)

25g Oxoid nutrient broth No. 2

75g glucose

Made up to 1 litre with distilled water

The addition of 1.5% w/v agar was used to prepare solid media. Approximately 20ml of medium was used per plate. All plates were allowed to set at room temperature after being poured and were then dried before use by being inverted and opened in a 37°C oven for 30 minutes.

Growth Conditions

All strains were grown at 37°C either in an incubator (solid media) or in a reciprocating water bath (liquid media). The water bath (Grant, type SS-30) was routinely set at 100 strokes per minute for growth of all *E. coli* and *S. typhimurium* strains except for the F′ donors used for gene mapping where gentler shaking was required and a rate of 50 strokes per minute was used. Growth of *Pseudomonas aeruginosa* requires a high level of aeration so that these cultures were shaken at 120 strokes per minute.

All strains were stored long term either lyophilised at 4°C or in 40% glycerol at -80°C as follows:-

Storage by lyophilisation

Paper labels bearing the strain identification written in pencil were placed in 100mm x 12mm soda glass test tubes. The tubes were then drawn out in the middle in an oxygen-gas

flame so that a Pasteur pipette was still able to pass through the constriction, plugged with cotton wool and autoclaved. The strain to be stored was grown up at 37°C with shaking in 20ml of an appropriate minimal medium plus supplements (see Table 2.2) and transferred to a sterile centrifuge tube when growth had reached late log phase (A_{660} =.7-.9 units, for measurement of absorbance see 2.3). The cells were pelleted by spinning at 8,000rpm for 10 minutes in an MSE HS18 centrifuge. The cell pellet was resuspended in 2ml glucose broth and 0.1-0.2ml of suspension transferred aseptically to each ampoule with a sterile Pasteur pipette. The cell suspension was frozen in liquid air and attached to a freeze drier until completely dry. The ampoule was then sealed while still attached to the freeze drier by heating the constriction in the ampoule with a small bunsen burner flame. The vacuum in the ampoule automatically seals each tube as the glass in the constriction softens. The ampoules containing lyophilised cultures were stored at 4°C where the cultures will remain viable indefinitely.

Ampoules were reopened by scoring round the outside with a glass cutting knife and pressing a red hot glass rod against the score mark. The cracking of the glass around the score mark enabled the end of the ampoule to be removed. The lyophilised cells were resuspended in 1ml of a suitable broth (usually L-broth) and transferred to a flask containing 20ml

of the same broth. The inoculated flask was incubated overnight at 37°C with shaking and then streaked onto minimal agar with supplements and incubated overnight at 37°C. A single colony from this plate was restreaked onto minimal agar with supplements and incubated overnight. A single colony from the second streaking was used to inoculate 20ml of a suitable liquid medium (see Table 2.2) which after overnight incubation at 37°C was stored at 4°C and used as a stock culture. The stock culture was restreaked on minimal medium agar plates lacking one supplement to check for strain purity.

Storage in glycerol

The cells to be stored were grown in a suitable complex medium (see Table 2.2) at 37°C overnight. Aliquots (0.5ml) of the culture were added to small sterile screw capped vials (2ml capacity) containing 0.5ml sterile 80% v/v glycerol in water. The vials were inverted several times (until the glycerol and culture were completely mixed) and placed in a -80°C freezer until required. To restart a culture stored in glycerol the vial was removed from the freezer and allowed to thaw at room temperature*. When thawed, a loopful (approximately 10ul) of cells was removed and used to inoculate 20ml of a suitable complex medium. The storage vial was returned to the freezer and the inoculated flask

* Note: cultures should not be thawed but inocula should be removed with a toothpick.

incubated overnight at 37°C. The culture was streaked twice and checked for strain purity as described for restarting lyophilised cultures (see above).

Stock cultures prepared from lyophilised or glycerol treated cells were maintained in liquid media and by streaking onto solid media and were subcultured every 3-4 weeks to maintain cell viability. These stock cultures provided inocula for the growth of cells used in experiments (see standard harvesting procedure, section 2.3)

2.3 STANDARD CELL HARVESTING PROCEDURE

Cells were grown by inoculating 20 or 50ml of medium with 0.5ml of a stock culture (see section 2.2) and incubating the cells at 37°C in a shaking water bath. The growth of the cells was followed by measuring the A_{660} of the culture with a Bausch and Lomb Spectronic 20 spectrophotometer. The relationship between cell density and absorbance was found to be linear up to an A_{660} of 0.5 units (data not shown), with a cell suspension of A_{660} =0.1 containing approximately $2x10^8$ cells (equivalent to 0.045mg cellular protein ml^{-1}). When the A_{660} of a culture had reached 0.2-0.4 the exponentially growing cells were harvested by filtration. Approximately 10-15ml of culture was filtered onto a Millipore cellulose

acetate filter (56mm diameter, pore size 0.45um) held in a Millipore filter holder. The filter apparatus was connected to a vacuum pump via a Buchner flask thus maintaining a negative pressure under the filter. The filter was washed twice with 20ml of 50mM potassium phosphate buffer pH7.2 at room temperature, removed from the filter apparatus and placed in a universal bottle containing a further 10ml phosphate buffer at room temperature supplemented with 0.05% (w/v) glucose. The bottle was vortexed to assist cell resuspension and the filter was removed. An aliquot of the suspension was taken for the experiment (routinely 5ml for an uptake assay) and the rest was used for measurement of the A_{660} as previously described.

2.4 GROWTH INHIBITION TESTS

The toxicity of peptide analogues was routinely tested using an agar plate inhibition zone assay. Two different types of this assay were used: 1) a lawn inhibition zone assay and 2) a radial streak inhibition assay. In 1) a solution of the test peptide was applied to a filter paper disc (blank sensitivity discs, 6mm, Oxoid Ltd.) in the centre of a plate on which a lawn of cells had already been spread. Routinely 100ul of a stock culture (see 2.2) containing approximately $2x10^8$ cells was spread onto a plate which was

then dried upside down in a 37°C oven for 20-30min. After drying, a filter disc was placed in the centre of the plate and 10ul of peptide solution was immediately added. The plates were then incubated upside down at 37°C for 18h (overnight). In 2) a fine platinum wire was used to streak cells, picked up by stabbing a fresh colony, from the centre of the plate outwards. Up to 32 streaks could be accommodated on a single plate in this way. Peptide was then added and the plates incubated as for method 1). After the overnight incubation the plates were inspected for growth inhibition. In method 1) this occurred as a circular zone around the filter disc while in method 2) the radial streaks started growing at a certain distance away from the filter disc. The distance of onset of growth away from the filter disc was measured in both cases. Both methods have different advantages; method 1) allows the effect of cell number on growth inhibition to be studied and facilitates the isolation of resistant cells (because more cells are included in the inhibition zone than in the radial assay). The frequency of occurrence of these mutants can also be calculated using method 1) because the number of cells plated out is known. Method 2) however allows many strains to be tested on a single plate and so is very cost effective both in terms of labour and materials. This method is ideal for screening large numbers of strains for the effect of several peptide analogues for example, when mutant isolates must be screened

to determine the nature of their mutation.

2.5 PEPTIDE TRANSPORT ASSAYS

Introduction

Four direct asays of peptide transport have been used in this study:- 1) Uptake of radioactively labelled peptides; 2) Dansyl Chloride assay; 3) Manual fluorescamine assay; 4) Continuous fluorescamine assay. These assays have already been discussed in section 1.4, therefore, only the practical details of each assay will be given here.

Uptake of radioactively labelled peptides - standard procedure

Glycyl-L-[U-^{14}C]Phenylalanine was obtained from The Radiochemical Centre, Amersham at a specific activity of 58mCi/mmol and at a radioactive concentration of 50uCi/ml. The concentration of Gly-Phe in this solution was approximately 4mM. The activity of the labelled Gly-Phe supplied was greater than was needed for the uptake assays and it was diluted as follows:- 10ul of radioactively labelled Gly-Phe was added to 990ul of 10.06mM "cold" Gly-Phe to give a final concentration of 10mM Gly-Phe with a

radioactive concentration of .5uCi/ml. This solution was then used as the stock solution. Exponentially growing cells were harvested and resuspended in 5ml 50mM potassium phosphate buffer pH7.2 as described in section 2.2. The cells were preincubated at 37°C for 10min before addition of 50ul of the radioactively labelled Gly-Phe solution previously prepared, to give final concentrations of 100uM Gly-Phe and 5nCi/ml radioactivity. Samples of cell suspension (500ul) were removed periodically (routinely every 30sec for 5min) and harvested on 25mm Whatman GF/C glass fibre filters held in a multiple holder manifold under vacuum and prewetted with 0.9% saline. The filters were washed with 0.9% saline (2x5ml), placed in plastic scintillation mini-vials and dried at 80°C for one hour. Soluene 350 tissue solubiliser (Packard Instruments Inc.) (1ml) was added and the vials were sealed with parafilm and incubated for 18 hours (overnight) at 45°C. NE260 micellar scintillant (Nuclear Enterprises Ltd) was added (5ml) and the vials were left at room temperature for 6 hours to reduce chemiluminescence before counting in a Packard Prias P L Tri-Carb liquid scintillation counter.

A sample (50ul) of incubation medium was removed at the end of each experiment, spotted directly onto a dry filter in a scintillation vial, dried and counted as previously described to determine the total number of counts present in

the incubation medium. Taking the sample at the end of the incubation period does not always provide an accurate estimate of the total number of counts present if, as under certain circumstances counts are lost during an assay through decarboxylation and release of $^{14}CO2$ (Payne and Nisbet, 1980a). In these cases samples should be taken at the beginning of the assay. Payne and Nisbet (1980a) have shown that the use of Gly-[U-^{14}C]Phe does not produce any significant loss of counts over the course of an assay and therefore in this case sampling at the end does provide an accurate determination of total counts present. A sample of cell suspension (500ul) was removed before the start of each assay and filtered as described. Labelled peptide diluted to 100uM in buffer (500ul) was passed through the filter and washed with 0.9% (w/v) saline (2x5ml). The filter was dried and counted as previously described. This control provided a zero time measurement and also gave an estimate of the adsorbtion of peptide to the cells and filter.

Dansyl chloride Assay- standard procedure (Payne and Bell, 1979)

Cells were harvested and resuspended as described in section 2.2, routinely 3ml of cell suspension was used per assay. After 10min preincubation at 37°C, peptide was added to the cell suspension to a final concentration of 100uM.

For measurement of peptide in the medium, samples (300ul) were periodically removed from the incubation medium (every 30s or 1min) using a 1ml syringe and were immediately freed from cells by filtration through Millipore cellulose acetate filters (13mm diameter, pore size 0.45um) in Swinnex filter holders. The incubation medium was pushed through the filter assembly into small (1ml) vials using a 10ml syringe full of air and was stored at -20°C. Cell extracts were prepared by removing 2ml samples in a 5ml syringe, filtered as described above and washed with 2x10ml distilled water at room temperature. The filter was then placed in a stoppered tube containing 2ml water and extracted for 15min in a boiling water bath. The boiled suspension was cooled, passed through a membrane filter to remove the cell debris and the filtrate stored at -20°C. Samples of incubation medium or cell extract (usually 100ul) containing up to 10nmol peptide were placed in Durham tubes (6x30mm) together with 5nmol diaminopimelic acid (DAP) standard and evaporated to dryness in a dessicator attached to an electric vacuum pump. The samples were usually added in several aliquots of 25-50ul with each aliquot being dried down before addition of the next one to prevent the sample splashing out when under vacuum. Sodium bicarbonate (200mM in deionised water, 20ul) was added to the tubes to adjust the pH to about 9, followed by 20ul dansyl chloride (2.5mgml^{-1} in Analar acetone). The tubes were sealed with parafilm and incubated at 45°C for

90min to allow dansylation to proceed to completion. The reaction mixture was then evaporated to dryness in vacuo and the solid residue redissolved in 10ul aqueous pyridine (1:1; v/v). Samples (5ul) were spotted near the corner of 15x15cm polyamide sheets (BDH Ltd, or Schleicher and Schuell Ltd) using microcap glass capillary pipettes and a warm air blower which dried the spots rapidly and prevented them from spreading. The polyamide sheets were placed in aluminium supports which enabled several sheets to be chromatographed at a time and ensured that the sheets did not touch each other during chromatography. The sheets were run in the following solvents until the solvent front had moved across approximately three-quarters of the plate (usually 45-60min) :- 1) 1st dimension, H_2O : formic acid (98.5 : 1.5, v/v); 2) 2nd dimension, acetic acid : toluene (10 : 90, v/v); 3) 2nd dimension, methanol : butyl acetate : acetic acid (40 : 60 : 2, v/v/v). A warm air blower was used to dry the plates completely between each change of solvent. Plates were often finally rerun in solvent 1) in the first dimension to improve the resolution of the spots. The fluorescent spots were visualised under long wave UV light and the spots identified and quantified by reference to the intensities of standard spots of the same substance after allowing for variations in the efficiency of the dansylation reaction indicated by the intensity of the standard DAP spot on each plate. Plates were photographed under long wave UV light using Ilfodata

HS23 film (Ilford) and a Wratten No. 3 filter (Kodak). Plates could be reused after washing in acetone : water : ammonia (50 : 46 : 4, v/v/v) for at least 3 hours.

Manual fluorescamine assay- standard procedure (Nisbet and Payne, 1979a,b)

All glassware used in this assay was cleaned by soaking for 24 hours in a detergent solution (1% Teepol), rinsing four times in tap water and twice in distilled water. Exponentially growing cells were harvested and resuspended as described in section 2.3. The cells were preincubated at 37°C for 10min before addition of peptide to a final concentration of 100uM. Samples (300ul) were periodically removed from the incubation medium (every 30s or 1min) with a 1ml plastic syringe and freed from cells by filtering through cellulose acetate filters (13mm diameter, pore size 0.45um) held in Swinnex filter holders. Incubation medium was pushed through the filter into small screw cap vials (1ml) with air from a 10ml plastic syringe. The filtrates were stored at -20° C until required. Samples (50ul) of filtrate containing 0-5nmol of peptide were added to 2.5ml 0.1M disodium tetraborate buffer, pH6.2 (made up in deionised water), in test tubes (100mmx12mm). Fluorescamine solution (500ul, 0.15mg ml^{-1} in Analar acetone) was added while the test tube contents were rapidly mixed using a vortex mixer. The

samples were left at room temperature for two minutes while the fluorescence stabilised. Samples were poured into a 1cm quartz cuvette and read in a Perkin Elmer 1000 fluorescence spectrophotometer (excitation wavelength 390nm, emission wavelength 485nm). Samples of suspension taken before addition of peptide and treated similarly to the experimental samples were used to determine the background level of fluorescence. Samples of known peptide concentration in buffer were used as references to enable the fluorescence readings to be converted to give peptide concentrations. The absolute fluorescence yield of a peptide can be calculated by reference to the fluorescence of a 1ug ml^{-1} solution of quinine sulphate in 0.1M H_2SO_4. Gly-Gly (100uM) gave a fluorescence yield of $4x10^{-1}$ that of the quinine sulphate reference solution when asayed under the standard conditions described (Nisbet, 1980)

Continuous flow fluorescamine assay - standard procedure (Payne and Nisbet, 1981)

Equipment and apparatus construction

The apparatus used in this assay is shown in Figure 1.4. The incubation vessel (usually a glass vial) was held in a thermostatically controlled water bath at 37°C with a magnetic stirrer placed beneath it so that the cell

suspension could be stirred during an incubation. The incubation buffer reservoir was also held in the water bath at 37°C whilst the reservoirs for channels B and C were at room temperature. The dead space in a Swinnex 25mm filter holder was reduced to the minimum possible which still allowed an adequate flow rate across the filter by inserting epoxy resin and filing down the filter support to one quarter of its original thickness. Fine grooves radiating from the centre were cut into the surface of the epoxy resin inserts to assist the flow through the filter. Whatman GF/C glass fibre filters (25mm) were used in this apparatus instead of cellulose acetate membrane filters because of their greater filtering capacity. A hypodermic needle (3in, 22 gauge) was attached to the upstream end of the filter apparatus to dip into the incubation medium and deliver the cells to the filter. The total dead volume for the filter apparatus was cut from several millilitres to 80ul by these various adaptations. This reduction in dead space reduced the amount of mixing of the incubation medium as it passed through the filter, improving the resolution of changes in peptide concentration. The pump used was a P-3 three channel peristaltic pump (Pharmacia) with 1mm internal diameter pump tubing supplied by Pharmacia. The other tubing used connecting the various components of the system was 1mm internal diameter tubing supplied by Esco Ltd.. The pump was routinely used on the maximum setting which pumped 0.9ml

min^{-1} through each channel. The mixing chambers were constructed from Technicon glass junctions having three outlet arms. Small resin coated metal spinners were inserted and trapped at the junction of the arms by sealing off the arm used for insertion with a flame. The spinner was driven by attaching the junction to a magnetic stirrer. The mixing chambers were connected via a delay coil to a Perkin Elmer 1000 fluorescence spectrophotometer fitted with a flow cell and debubbler. The delay coil (5.7m of tubing) allowed the fluorescence to stabilise and also allowed the small bubbles produced during the mixing of the buffered incubation medium and fluorescamine solution to aggregate into bubbles large enough to be removed by the debubbler.

Standard procedure

The buffer used in channel B for peptide transport assays was 0.2M citrate phosphate buffer pH5.8 while for amino acid transport assays 0.1M disodium tetraborate pH9.4 was used. Fluorescamine (channel C) was used in analar isopropanol (.15mg ml^{-1}). Exponentially growing cells were harvested and resuspended in 50mM potassium phosphate buffer as described in section 2.3. Routinely 5ml of cell suspension was placed in the incubation vessel, providing enough cells for an assay of approximately 5 min (if longer assays were required the volume of cell suspension was increased accordingly). The

cell suspension was preincubated at 37°C for 10min with stirring. During the preincubation period the filter assembly was opened and the filter used in the previous assay removed. The assembly was then washed with distilled water and a new filter inserted with the assembly containing enough water to ensure that no air was trapped under the filter, as this interferes with the flow of cell suspension during the assay. Resuspension buffer at 37°C was pumped through channel A for the remainder of the preincubation period (4-6min) to obtain a steady baseline on the output trace. At the start of the assay peptide was added to a final concentration of 100uM, the pump was momentarily stopped and the needle transferred to the incubation vessel. The pump was restarted and the assay continued until all the cell suspension had been drawn from the incubation vessel when the needle was rapidly transferred to the incubation buffer reservoir to prevent air from entering the system. Incubation buffer was pumped through the system until a steady baseline was obtained again. The output from the fluorimeter was recorded on a Servoscribe chart recorder, or in later experiments on a floppy disc (Maxell MD2) using a Research Machines 380Z microcomputer interfaced with the fluorimeter (see section 2.6). When cell suspension alone was pumped through the system no change in the baseline was observed so that this control was not routinely required. A solution of the peptide used in the assay (of known

concentration in incubation buffer) was run through the system to provide a reference fluorescence yield enabling experimental fluorescence yields to be converted to peptide concentrations. It was not possible to determine the absolute fluorescence yield in this system using quinine sulphate in sulphuric acid (see manual fluorescamine assay) as the sulphuric acid might attack the components of the pumping system. A solution of 100uM Gly-Gly in incubation buffer, when assayed by both manual and continuous fluorescamine standard procedures, gave a 3x higher fluorescence yield with the continuous assay (Nisbet, 1980). The slope of the output trace was measured (i.e. the change in fluorescence yield per unit time) and by converting the values for fluorescence yield to peptide concentration the rate of peptide uptake was calculated.

2.6 COMPUTER DATA LOGGING AND HANDLING

Introduction

One of the major advantages of the continuous flow fluorescamine assay over the manual fluorescamine assay is the much greater volume of data generated by each experiment. The Perkin Elmer 1000 fluorescence spectrophotometer used in this study generates one reading every 0.64sec (which is

itself an average of 8 readings taken every 0.08sec) and to fully utilise this output a method of recording more precise than that provided by a chart recorder was required. The output from the fluorimeter was in a digital format so that the logical step was to use a computer to log the data enabling every reading to be precisely recorded. Once the data have been recorded the computer can be used to analyse it, providing for example transport kinetic parameters which would have been laboriously and less reliably provided by manual calculation. The microcomputer and peripherals used were as described in section 2.1.

Data logging

The data logging was performed by the program D-LOG. When D-LOG is run it creates a file in which to store the data and allows a description of the conditions used in the assay to be recorded at the beginning of the file. The logging process is then started by pressing any key on the keyboard so that at the beginning of an assay when there are several operations for example, stopping and starting the pump and adding peptide, all to be performed in a short time, starting the data logging process is a simple single action. The program then records every reading generated by the fluorimeter and also sends it to the printer to provide a hard copy of the assay as it is proceeding. The data logging

is stopped at the end of an assay by pressing two specific keys simultaneously, preventing accidental stoppage of the program by the inadvertant pressing of a key. The program has several other facilities: 1) The recording can be stopped and all the data collected during that run deleted while still retaining the file description allowing repeats of assays which were not completed because of for example, air blocking the filter, without having to re-enter the file description; 2) The file description can be amended after data recording has been completed so that remarks can be added; 3) The whole file can be erased when a run is incomplete and no repeat run is required. The file for a routine 5min assay occupies approximately 3 kilobytes (kb) of memory so that each floppy disc with 36kb of memory on each side can accommodate around 24 assays.

Data processing

The data produced by D.LOG were processed in two stages. The first stage was to convert the data points which represented the fluorescence yield at a particular time during the assay to peptide uptake rates and peptide concentrations. The second stage (if required) then used the uptake rates and concentrations to calculate Michaelis Menten kinetic parameters. When only an overall transport rate was required from the assay data, only the first stage of

processing was required.

GRAPH

The first stage of data processing was accomplished by the program GRAPH. This program displayed the data from a file together with the file description on the monitor. The data was displayed on axes of fluorescence (x-axis) and time (y-axis) with a variable scale expansion of the y-axis. The variable scale expansion was accomplished by combining two or more adjacent data points to produce traces one half the length of the original (if pairs of data points were combined) or shorter (if more than two data points were combined to produce each new data point). A trace at maximum scale expansion i.e. each individual datum point shown, was too long to fit on one monitor screen width so that to see the whole trace at once, a lower scale expansion was used (routinely 3 or 4 data points were combined to produce each point displayed). The scale expansion was set at the beginning of each run of GRAPH when the program prompted for the value to be used. When the trace was too long to fit on one screen width it could be moved across the screen in steps by a set number of data points each time (specified at the beginning of the program). The sideways movement of the trace could be stopped and restarted at any time by specific key actions. The calculation of slopes (which can be

converted to transport rates) and concentrations was achieved by the use of two movable pointers generated by the program. The pointers could be moved independently along the trace by key actions and when in position another key action prompted the program to calculate the average slope of the trace between the pointers (in fluorescence units per min) and the height (fluorescence yield) of the midpoint and display the results on the monitor screen. A single estimate of slope and height over the whole trace was sufficient to determine the overall transport rate. For calculations of kinetic parameters a series of estimates of slope and height were made over portions of the trace and used to calculate the transport rates (v) and peptide concentrations (s). Two additional facilities of GRAPH were used in this process, a data correction subroutine and a repeated estimation subroutine. A full explanation of these facilities is provided in 4.5.

MICMEN

MICMEN is a program which calculates the Michaelis Menten kinetic parameters from input values of peptide uptake rate (V) and peptide concentration (S). The program works by calculating the deviation sum of squares (dss) of the data from the curve produced by the Michaelis Menten equation

$$V = \frac{VmaxS}{Km + S}$$

and changing the values for Vmax and Km through successive iterations until the smallest value of the dss is found (the least squares method). The data was prepared in a file with all the S values in one column and the corresponding V values in the next column. The file was terminated by a negative value. When the program is run it initially plots the data on a Lineweaver-Burke plot (V against V/S) and calculates values for Vmax and Km. These values are then used to calculate the search length, reduction factor and minimum search length (see later) as well as providing a start point for the fitting process.

The way in which the program moves towards the best fitting Michaelis Menten parameters can be visualised by assuming that the Vmax and Km are the x and y axes of a two dimensional plot. The program starts in a position decided by the Lineweaver-Burke plot and calculates the deviation sum of squares (dss) for those values of Km and Vmax. The program then calculates the dss values for the four points vertically or horizontally z units away from the start point where z = the search length specified at the beginning of the program. Whichever of the four points produces the lowest dss is used as the base for the next four points. In this

manner the program arrives at a rough estimate of the best fitting values for Vmax and Km when the base point is surrounded by points with greater dss values. At this stage the distance over which the four points are taken is reduced by the reduction factor as specified at the beginning of the program run and the best fit parameters are searched for as before. This reduction in search distance followed by the search occurs repeatedly until the distance used reaches the minimum search length also specified at the beginning of the program run. When this point is reached the program prints out the final parameters Vmax, Km as well as an analysis of variance for the data set. During the search process the data being used are displayed on the monitor and the hyperbola produced by the values for Km and Vmax being used for the base point at that stage is overlaid on them. In this way the change in the shape of the curve can be monitored as the program proceeds towards the best fit parameters. The program can also provide a graph of Vmax against Km with various confidence limits drawn as contours. This plot illustrates how much variation in the parameters is possible without exceeding 95% or 90% confidence limits and is useful in determining the degree of overlap between different estimates of Michaelis Menten parameters. A worked example of the data gathering and processing procedure is shown in Appendix A.

2.7 PEPTIDASE ASSAYS

Intracellular peptidase assay

The intracellular peptidase activity was measured by incubating peptide solutions with crude cell extract, removing samples periodically and quantifying the levels of peptide and amino acid product present by thin layer chromatography.

Standard assay

Cells were grown in A+C medium (see section 2.2) in 50ml batches and were harvested when growing exponentially (A_{660} =.3-.5) as described in section 2.3. The cells were washed in 20mM potassium phosphate buffer pH7.6 containing 1mM cobalt sulphate (2x20ml) and were resuspended in a further 10ml of buffer in 50ml polypropylene centrifuge tubes (MSE) to a cell density of $3\text{-}5\times10^{9}$ cells ml^{-1}. After cooling on ice for 10min the cells were disrupted by sonication using an MSE Soniprep 150 sonicator in 5x1min bursts with 2min cooling on ice between treatments. The sonicate was centrifuged in an MSE HS18 centrifuge (8x50ml rotor) for 20min at 5,000rpm and 4°C to remove the cell debris. The supernatant solution was stored at -20°C until required. Crude extract was stable at -20°C over a period of at least

several weeks, in addition, the total volume of extract was divided into 3ml aliquots so that each sample was only thawed once before it was used in an assay. The protein content of each extract was estimated by the Lowry assay (Lowry et. al., 1951).

Cell extract (2.775ml) was equilibrated in 100mm x 12mm test tubes at 37°C for 10min before 225ul of peptide was added to give a final peptide concentration of 7.5mM. Samples (300ul) were removed at 0, 10, 20, 40, 60, 100, 150 and 200min after the addition of peptide and put in small vials (1ml) already containing 10ul of concentrated HCl to stop any further peptidase activity. The vials were shaken and then stored at -20°C until the samples were chromatographed. Thin layer plates were prepared by making up a 60% w/v slurry of Kieselgel 60G silica gel (Merck) in water with vigorous shaking for 90sec. Glass plates (20cm x 20cm) were held in a horizontal rack (Shandon Uniplan leveller No.2810) and the silica gel was spread over them immediately after mixing using a Shandon adjustable spreading device (No.2818) set to give a layer 0.25mm thick. The plates were allowed to dry at room temperature for 30min and were activated by heating to 110°C for 10min. Samples (10ul) were loaded onto the thin-layer plates, 2cm from the base of the plate, using Microcap glass capillary pipettes and were allowed to dry at room temperature for 30min before

chromatography. The plates were run in butanol : acetic acid : water : pyridine (75:15:60:50; v/v/v/v) until the solvent front had reached three-quarters of the way up the plate. The plates were then dried at 50°C for 30min and the spots were visualised using a spray of acetone : acetic acid : water (100:10:5; v/v/v) containing 1% w/v ninhydrin and 0.1% w/v cadmium acetate. The rates of peptide cleavage were determined by comparing the intensities of the spots with peptide and amino acid spots of known concentration run on the same plate.

Aminopeptidase N assay

Introduction

Aminopeptidase N is a periplasmic enzyme which is able to hydrolyse amino acid p-nitroanilides to amino acid and p-nitroaniline (Lazdunski et al., 1975). Because p-nitroaniline absorbs at 415nm while the amino acid p-nitroanilide does not, the activity of aminopeptidase N can easily be measured by monitoring the absorbance of a cell suspension at 415nm (Lazdunski et. al., 1975). Because amino acid p-nitroanilide can penetrate to the periplasmic space and p-nitroaniline can diffuse out again, whole cells can be used, which simplifies the assay and avoids the release of intracellular enzymes which might also have hydrolytic

activity towards the substrate.

Standard procedure

Exponentially-growing cells were harvested and resuspended as described in section 2.3 except that the washing and resuspension buffer was 100mM disodium tetraborate pH7.2. To prevent nitrogen, phosphate or carbon starvation, 0.25mM ammonium chloride, 2mM potassium phosphate and 0.5% w/v glucose were added to the buffer. Samples of resuspended cells (2.9ml containing approximately $5x10^8$ cells ml^{-1}) were placed in 100mm x 12mm test tubes (Pyrex, thin walled for use in the spectrophotometer) and were preincubated at 37°C for 15min. Alanyl p-nitroanilide (Sigma) was added (100ul, 45mM in water) to give a final concentration of 1.5mM. The cells were incubated at 37°C with the production of p-nitroaniline being followed by measuring the change in absorbance at 415nm in a Bausch and Lomb Spectronic 20 spectrophotometer. Each tube was placed in the spectrophotometer for direct reading of absorbance with the zero time reading acting as the blank control. A standard curve of absorbance against concentration was constructed by measuring the absorbance at 415nm of p-nitroaniline solutions of known concentration made up in resuspension buffer (3ml) in tubes previously blanked with resuspension buffer only. The rate of cleavage of alanine p-nitroanilide was calculated by using the standard

curve to convert experimental absorbance readings to p-nitroaniline concentrations.

2.8 GENE MAPPING

Introduction

The approximate locations of the mutations causing loss of transport activity were mapped by F′ factor conjugation. F′ factors are derivatives of the F sex factor, produced by aberrant excision of F from the chromosome of an Hfr cell. In Hfr (high frequency donor) cells the F factor is integrated in the chromosome and occasionally during F excision, portions of the chromosomal DNA adjacent to the site of F factor integration are also excised. The genes acquired by such an F factor derivative (F′) are transferred with high frequency to F factor lacking (F) recipients along with the rest of the F′ episome. A large number of such F′ factors have now been isolated and characterised. If a recipient strain containing a mutationally inactivated gene is mated with a selection of F′ donor strains containing different sections of chromosomal DNA, complementation can occur when the F′ contains the gene mutated in the recipient. Given that the region of chromosomal DNA carried by each F′ is known, then the mutant gene can be mapped to a particular

region of the chromosome. This is the method employed here to map the genes coding for the peptide permeases, using enhanced ability to utilise Lys-Lys or Lys-Lys-Lys as lysine sources as an indication of restoration of a particular peptide permease.

Standard procedure

Donor and recipient cells (see Table 2.1) were grown for 18hr (overnight) in 20ml L-broth (see section 2.2). These overnight cultures were used to provide inocula for growth in fresh L-broth. Recipient strains were subcultured by inoculating 50ml of L-broth with 1ml of overnight culture. Donor strains were subcultured in groups of five, so that later on when matings were started, the number of crosses to be set up at one time was manageable. Each group was inoculated one hour apart using 200ul of overnight culture into 20ml of L-broth. The recipient cells were incubated at 37°C in a reciprocating water bath (Grant) at 100 strokes per min and allowed to grow to stationary phase. The donor cells were also incubated at 37°C but were shaken less rapidly (50 strokes per min) and were allowed to grow until their A_{660} had reached 0.2-0.4 (see 2.3). Donor cells (1ml containing $2\text{-}4\times10^{8}$ cells) were gently mixed with recipient cells (1ml containing 10^{9} cells) in boiling tubes and left for 2.5hr at 37°C with no shaking, to allow conjugation to occur. The

mating mixtures were filtered onto Millipore 25mm cellulose acetate filters (pore size 0.45um) using a multiple holder manifold connected to a vacuum pump, maintaining a negative pressure under the filters. The filters were washed with potassium phosphate buffer pH7.2 (2x10ml) and resuspended in a further 2ml of the same buffer to give a cell density of approximately $1x10^9$ cells ml^{-1}. Samples of cell suspension (50ul) were plated out onto half an agar plate containing either minimal medium (A+C, see 2.2) or minimal medium supplemented with Lys, Lys-Lys, or Lys-Lys-Lys (all $10ugml^{-1}$). The plates were left at room temperature until the suspension buffer had been absorbed (15min) and then incubated at 37°C. The plates were inspected for growth at least every 3 hours for 18 hours and the presence of background growth and faster growing colonies was noted.

Discussion

If the F′ donors are shaken too vigorously when being cultured, the shearing effect of the solution drastically reduces the number of sex pili present on the cell surface so that conjugation is a much less frequent event, however some shaking is of benefit as it enhances the aeration of the culture. For these experiments the donor cells were shaken at 50 strokes per minute instead of the usual 100 strokes per minute as a compromise between the level of shaking required

for full aeration and the need to prevent loss of pili. The conjugation mixture must not be shaken at all as the bond formed by the pili between the mating cells is fragile and any movement during this process reduces the frequency of conjugation.

Because of the overlapping specificities of the three peptide permeases the possibility exists that the loss of one or even two of them may not entirely prevent the growth of a deficient mutant on Lys-Lys or Lys-Lys-Lys so that plating out on a medium only allowing growth of exconjugants was not possible. The rate of growth of dpp and opp mutants is however slower than that of the wild type on Lys-Lys or Lys-Lys-Lys respectively as lysine sources. To maximise this difference in growth rates a peptide concentration just enabling maximal growth to occur in the wild type was selected by comparing growth on plates containing different peptide concentrations. By examining the plates at least every 3 hours for up to 10-15 hours it was possible to see which mating mixtures contained cells with enhanced growth rates by the appearance of colonies growing faster than the background (50-1000 colonies per plate). After prolonged incubation (over 15 hours) the colonies tended to be masked by growth of the background parental cells except in the case of the triple permease deficient mutant; no growth took place on the peptides unless a transport system was present on the

F′ so that identification in these cases was much simpler. The frequency of conjugation can be calculated from the number of conjugants and parental cells. The frequency calculated in this manner varies from 1 in 10^3 to 1 in 10^5 cells indicating that under the conditions used conjugation was not very frequent. A possible explanation for this low frequency may be that the donor and recipient cells used were different types of Escherichia coli (K12 and W) respectively). Mating between other different types of Escherichia coli is known to be less frequent than mating between the same type (Hayes, 1968) so that in this case it seems likely that the frequency would also be low.

2.9 ELECTROPHORETIC RESOLUTION OF OUTER MEMBRANE PROTEINS

Introduction

Crude cell envelopes were prepared by high speed centrifugation of cell sonicates, these envelope preparations were then separated into inner and outer membrane fractions by their differential solubility in Triton X-100 (Schnaitman, 1971). Discontinuous SDS-polyacrylamide gel electrophoresis was used to separate individual protein species.

Outer membrane fraction preparation

Cells were grown in L-broth (50ml) and harvested during the late logarithmic phase by centrifugation at 8,000 r.p.m. for 10min at 4°C in the 8x50 ml rotor of an MSE HS18 centrifuge. The cells were washed with 10ml of cold M9 minimal medium and resuspended in a further 2ml of cold M9. After sonic disruption for 3x20 second periods, with cooling on ice for 2 minutes between each period, in an MSE soniprep set at an amplitude of 15um, any unbroken cells and large fragments were removed by centrifugation at 1500g for 10min. The supernatant fluid was then centrifuged at 100,000g for 30min in an MSE prepspin to recover the cell envelopes. The cell envelope pellet was resuspended in 2ml 2% Triton X-100 in 10mM Tris-HCl pH7.5 and incubated at 37°C for 30min. The suspension was then centrifuged at 100,000g for 30min to recover the insoluble outer membrane fraction. The outer membrane pellet was resuspended in 50ul of 50mM Tris-HCl pH7.5 and the protein content measured by the spectral method of Whitaker and Granum (1980).

Polacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out as described by Lugtenberg et. al., 1975. The compositon of the running and stacking gels was as follows:

Running gel

11% (w/v) acrylamide

0.2% (w/v) methylene-bisacrylamide

0.375M Tris-HCl pH8.8

0.2% (w/v) SDS

0.25mgml^{-1} ammonium persulphate

0.2% (v/v) TEMED

(50ml)

Stacking gel

3% (w/v) acrylamide

0.2% (w/v) methylene-bisacrylamide

0.125M Tris-HCl pH6.8

0.1% (w/v) SDS

0.25mgml^{-1} ammonium persulphate

0.2% (v/v) TEMED

(10ml)

The solutions were degassed before the addition of the SDS, ammonium persulphate and TEMED.

The gels used were 18cm x 15cm x 1.5mm with the bottom 13cm being running gel and the top 2cm being stacking gel. To ensure a smooth interface between the running and stacking gels, distilled water was layered onto the top of the running gel immediately after it had been poured, using a syringe barrel and fine needle. After the running gel had set the water was poured off before the stacking gel was added. The buffer for both electrodes contained 0.025M Tris, 0.19M glycine and 0.1% (w/v) SDS and had a pH of 8.3. Protein samples were prepared in 0.0625M Tris-HCl pH6.8, containing SDS (2%, w/v), glycerol (10%, v/v), bromophenol blue (0.001%, w/v) and 2-mercaptoethanol (5%, v/v). Samples contained approximately $1mgml^{-1}$ protein and were boiled for 5min immediately before application to the gel. Routinely 20ul (approximately 20ug protein) was applied to each slot. Bromophenol blue was added to the upper electrode reservoir just before electrophoresis commenced and was used as a tracking dye. Electrophoresis was carried out using a constant current of 8mA at room temperature so that the bromophenol blue took 16-18hr to reach the bottom of the gel. Gels were stained in 0.025% (w/v) Kenacid blue in methanol : water : glacial acetic acid (50:43:7, v/v/v) for 24 hours with gentle shaking and destained in the same solution without Kenacid blue until the bands were at their most clearly visible (3-6h). Gels were photographed using a Kodak CC 23A filter before drying down in a Shandon vacuum gel

drier for permanent storage.

The following standard proteins were used as molecular weight markers:- bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), concanavilin A (26,000 daltons) amd myoglobin (17,200 daltons).

2.10 MEASUREMENT OF ALKALINE PHOSPHATASE ACTIVITY

Introduction

Under alkaline conditions alkaline phosphatase cleaves p-nitrophenyl phosphate to p-nitrophenol and phosphate. The p-nitrophenol absorbs at 415nm so that the production of p-nitrophenol can be monitored using the increase in absorbance at that wavelength.

Standard procedure

50ml batches of cells were grown in 0.2M TrisHCl pH8.0 containing 3mM K_2HPO_4, 50ugml^{-1} lysine and 0.5% (w/v) glucose. When their A_{660} reached 0.4, the cells were harvested as described in section 2.3 and resuspended in 100mM TrisHCl pH8.0 containing 0.5% (w/v) glucose, 50ugml^{-1} lysine and 0.1mM phosphate, to an A_{660} of 0.3-0.4. 1ml

aliquots of cell suspension were placed in cuvettes in a thermostatically controlled cuvette holder in a Gilford spectrophotometer and allowed to equilibrate to 37°C for 10 min before addition of p-nitrophenylphosphate substrate to a final concentration of 1mM. The A_{415} of the suspension was then recorded on a chart recorder for up to 120min.

3 ISOLATION AND CHARACTERISATION OF PEPTIDE PERMEASE DEFICIENT MUTANTS IN ESCHERICHIA COLI W

3.1 INTRODUCTION

Small peptides have been shown to be transported into *E. coli* via at least three transport systems, the dipeptide permease (*dpp*), the oligopeptide permease (*opp*) and a third system (*opt*) (Payne, 1980). Based mainly on the result of growth tests the *dpp* was reported to be specific for dipeptides (Payne, 1968; DeFelice *et al.*, 1973); the *opp* to be broadly active towards both di- and oligopeptides (Payne and Bell, 1979) and the *opt* to have a restricted specificity for a few tripeptides (Barak and Gilvarg, 1975; Naider and Becker, 1975). Recent results (Alves and Payne, 1980), measuring peptide transport directly, indicate that these specificities may have to be reassessed.

Peptide permease deficient mutants have been widely used in the study of peptide transport. Mutants lacking the *opp*, the *dpp* or both have been isolated (Payne, 1968; DeFelice *et al.*, 1973; Vonder Haar and Umbarger, 1972). Mutants resistant to trithreonine, which are presumably defective in *opt*, have also been isolated (Barak and Gilvarg, 1975).

Mutants are normally isolated using spontaneously occurring resistance to toxic peptides as a selection procedure. The mechanisms by which cells can acquire resistance have been discussed in section 1.3, however it is pertinent to summarise them here as a means of explaining the strategy adopted for characterising the mutants isolated in this study. Resistance to toxic peptides can be gained by any of a number of mechanisms, for example, 1) resistance to the toxic moiety per se; 2) decreased peptidase activity, preventing the release of the toxic moiety within the cell interior, or increased peptidase activity, if the intact peptide is the toxic species (as in the case of triornithine); 3) decreased transport ability, lowering the internal pool of toxic moiety (intact peptide or hydrolytically released residue) to below its inhibitory threshold. The experiments performed to characterise the mutants were therefore designed to determine which of these mechanisms was responsible for the increased resistance observed. With regard to 1), cross-resistance tests can show whether the same moiety attached to another peptide is still toxic; 2) can be investigated by direct measurement of peptidase activities in wild type and mutant strains; 3) can be directly measured using the sensitive fluorescence assays recently developed (Nisbet and Payne, 1979a; Payne and Nisbet, 1981).

There has been much interest in the possibility of using peptides as a means of introducing impermeant toxic moieties into bacterial cells using the "smugglin" principle (Matthews and Payne, 1975a). Consequently new peptide mimetic antibacterial agents were synthesised, some of which appeared to be transported more or less specifically via one or other of the peptide transport systems. This raised the possibility of isolating novel peptide permease deficient mutants. These mutants could then be used in combination with the fluorescence uptake assays to determine the specificities of each permease. With this information future "smugglin" antibiotics could be rationally designed to give maximum efficiency of uptake via more than one uptake system thus minimising the risk of resistance occurring through loss of permease activity. Several of the novel peptide mimetic antibacterial agents have been used in this study to isolate mutants deficient in dpp, opp and opt. This is the first report of a mutant totally defective in peptide transport. These mutants have been used in conjunction with the manual and automated fluorescamine assays (Nisbet and Payne, 1979a; Payne and Nisbet, 1981) to determine the specificities of the three peptide permeases. The approximate locations of the genes coding for each permease have been mapped by F′ donor conjugation.

3.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The strains used are shown in Table 2.1 and the growth media in Table 2.2. Growth conditions are described in section 2.2.

3.3 ISOLATION OF MUTANTS RESISTANT TO TOXIC PEPTIDES

Introduction

Mutants defective in *opp* are easily isolated by selection for resistance to triornithine (Payne, 1968). Mutants defective in *dpp* are more difficult to isolate presumably because dipeptides can be transported via the *dpp* and *opp* and as *opp* mutations occur at an unusually high frequency of approximately 1 in 10^5 cells (Gilvarg and Levin, 1972; Barak and Gilvarg, 1974; section 3.4) they will usually be picked up rather than *dpp* mutations.

During studies of a range of peptide mimetic antibacterial agents on wild type and *opp* strains (see Table 3.1), it was noted that several toxic dipeptides were almost as effective against an *opp* strain as a wild type strain. This indicated that these peptides might be transported predominantly via the Dpp and that they might be used to isolate mutants

Table 3.1 Sensitivity of wild type and opp strains of E. coli W to toxic peptide mimetics.

Peptide used	wild type	opp
Ala-Ala-aminoxyAla	34(10nmol)	12
Pro-Ala-aminoxyAla	28	12
BocPro-Ala-aminoxyAla	>(2.5nmol)	0
Ala-hydrazinoAla	43	0
Ala-Ala-hydrazinoAla	35	0
Ala-aminoxyAla	36	34
Pro-aminoxyAla	18	17
Val-aminoxyAla	34	31
Lys-aminoxyAla	33	33

All values are inhibition zone diameters in mm.

100nmol of all peptides used per plate unless stated.

> whole plate inhibited.

For structures of these peptide mimetics see Appendix B.

Nomenclature of the structures is described in Morley et al., 1983).

defective in dpp. The most toxic of the dipeptides, Ala-aminoxyAla (for structure see Appendix B) was used to isolate what was initially thought to be a dpp mutant, but which has later been shown to have, in addition, a partially defective opp (strain PA0107). Using different toxic peptides, a mutant showing complete resistance to peptides believed to use opp (strain PA0119) was isolated from PA0107 and a further mutant totally lacking peptide transport activity (PA0122) was isolated from PA0107. An opp mutant (PA0112) was isolated from the wild type by selection with Ala-Ala-aminoxyAla and an opp dpp double mutant (PA0113) from PA0112 by selection with Ala-aminoxyAla.

Methods

Most of the mutants were isolated by the agar plate inhibition zone test described in section 2.4. A dpp strain proved difficult to isolate using this method, therefore a selection strategy based on enrichment of a culture with dpp cells, by growth in the presence of Ala-aminoxyAla, was followed (see figure 3.1). The other defective mutants were isolated using the toxic peptides shown in Table 3.2). Colonies of presumptive transport deficient mutants were purified by streaking twice and then were tested with the peptide originally used for selection, to confirm an increase in resistance.

Figure 3.1 The procedure used to isolate putative dipeptide permease deficient mutants, using resistance to Ala-aminoxyAla as a selection.

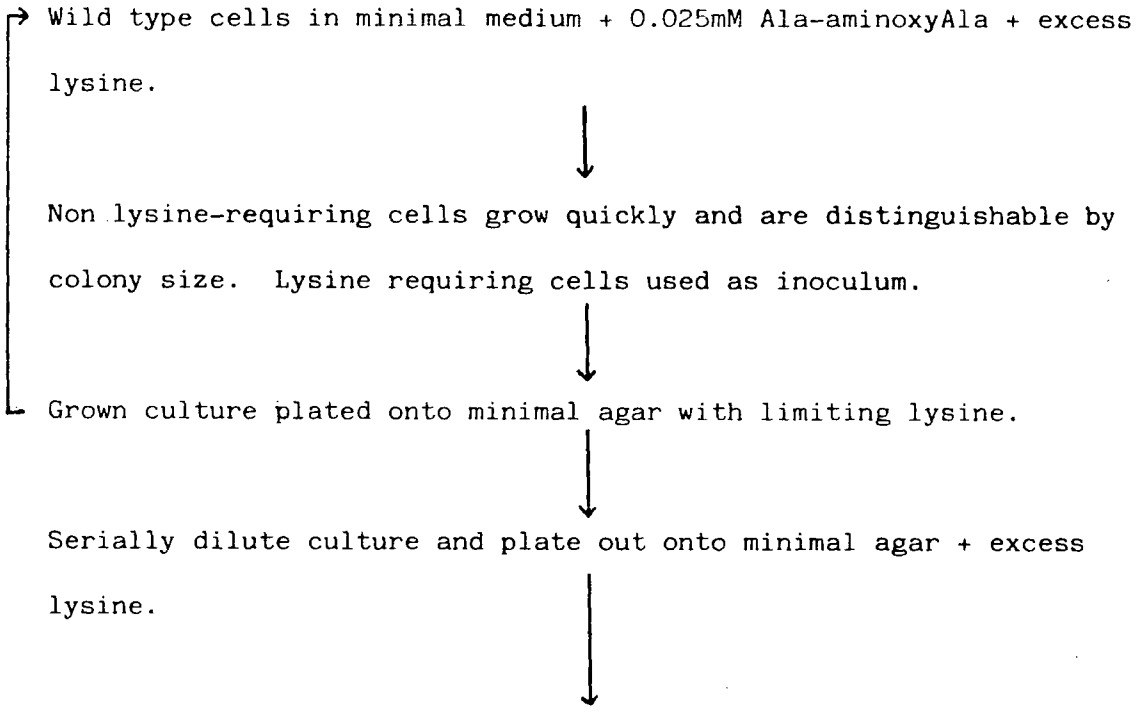


Table 3.2 Toxic peptides used to select for peptide transport deficient mutants.

Mutant selected for	Peptide used
dpp (from wild type)	Ala-aminoxyAla
opp (from wild type)	Ala-Ala-aminoxyAla
dpp opp (from dpp)	Ala-Ala-aminoxyAla
opp dpp (from opp)	Ala-aminoxyAla
dpp opp opt (from dpp opp)	Ala-AlaP

Ala-Ala-aminoxyAla used at 20nmol per plate.

Ala-aminoxyAla and Ala-AlaP used at 200nmol per plate.

For structures of these peptide mimetics see Appendix B.

Results

The results of the initial screening of toxic peptide mimetics against wild type and opp strains is shown in Table 3.1. It was decided to use Ala-aminoxyAla to select for dpp mutants as it was the most toxic of the aminoxy dipeptides tested (for structures of the peptide mimetics used see Appendix B).

Discussion

In initial attempts to isolate dpp mutants using Ala-aminoxyAla in the inhibition zone assay only opp mutants were isolated, presumably because Ala-aminoxyAla, although transported significantly by the Dpp, also goes in via the Opp and as opp mutants occur relatively frequently (Gilvarg and Levin, 1972; Barak and Gilvarg, 1974; section 3.4) they will be the ones which tend to be picked up. More recent work (J.W. Payne, personal communication) has shown that more than one transport system must be inactivated if a significant decrease in sensitivity to Ala-aminoxyAla is to be observed in an inhibition zone assay.

The enrichment procedure adopted to try to isolate a dpp strain attempted to provide conditions where dpp cells would have a maximal selective advantage over either wild type or

opp cells using a concentration of Ala-aminoxyAla which is just inhibitory towards wild type cells. It was hoped that under these conditions dpp cells would outgrow either wild type or opp cells and thus increase as a proportion of the overall cell population. After two rounds of the enrichment procedure, cells with a marked increase in resistance to Ala-aminoxyAla were obtained. However this procedure also makes it possible to isolate cells containing more than one mutation, because of the large number of cells involved and the repeated selections.

Double mutants lacking both dpp and opp were isolated either by selecting for loss of opp from a dpp strain or by selecting for loss of dpp from an opp strain. These two mutants were then to be tested for peptide uptake activity and for antibiotic sensitivity to determine whether the order of acquisition of mutations affects the final phenotype (assuming that the mutations affecting each strain are of a similar type).

3.4 FREQUENCY OF OCCURRENCE OF SPONTANEOUS RESISTANCE TO TOXIC PEPTIDES

Introduction

There have been reports (Gilvarg and Levin, 1972; Barak and Gilvarg, 1974) that loss of the opp occurs at a higher frequency than that determined for many other types of spontaneous mutation (1 mutant per 10^5 cells for opp , compared with a typical value of about 1 mutant per 10^7 cells). Such a high frequency of acquisition of resistance has obvious implications for the design of antibiotics whose entry into the cell is only via the oligopeptide permease. To date most mutants lacking opp have been isolated using resistance to triornithine as a selection procedure. To check that triornithine itself was in no way responsible for the high rate of mutation to opp , the rate of occurrence of opp mutants using several other toxic tripeptides as selective reagents was tested. It was also of interest to measure the rates of occurrence of mutants deficient in dpp and opt to determine whether all peptide transport deficiency mutations occur at a high rate or whether opp mutations are atypical.

Methods

Agar plate inhibition zone assays were performed as described in section 2.4. The number of resistant cells occurring in the inhibition zone were counted. The number of cells plated out per unit area can be calculated from the total number of cells plated and the total area of the plate.

The inhibition zone area multiplied by the number of cells per unit area gives the number of cells in the inhibition zone. The number of resistant cells divided by the number of cells initially plated in the inhibition zone gives the mutation rate.

Results

The frequency of mutations in *opp*, *dpp* and *opt* in wild type, *opp* and *dpp opp* strains respectively were calculated. The results obtained are shown in Table 3.3.

Discussion

The rate of loss of *opp* is independent of the peptide used for selection and was found here to be at 1 in 2-4 x 10^5 cells, similar to the rates previously reported (Gilvarg and Levin, 1972; Barak and Gilvarg, 1974). Loss of *dpp* or *opt* occurs at a typical frequency of 1 in 5 x 10^6 to 1 in 10^7 cells. As *dpp* and *opt* mutations occur less frequently than *opp* mutations, peptide antibiotics designed to be transported primarily via the *dpp* and/or *opt* should be less likely to encounter problems with bacterial resistance than those primarily using the *opp*.

Table 3.3. Frequencies of mutation in peptide permeases.

Peptide	Mutation	Frequency
Orn-Orn-Orn	opp	1 in $4x10^5$
Ala-Ala-aminoxyAla	opp	1 in $6x10^5$
Gly-Gly-Norleu	opp	1 in $5x10^5$
Ala-aminoxyAla	dpp	1 in $7x10^6$
Ala-AlaP	opt	1 in $9x10^6$

For structures of peptides see Appendix B.

3.5 CROSS-RESISTANCE OF PEPTIDE-RESISTANT MUTANTS TO OTHER TOXIC PEPTIDES

Introduction

As described in section 3.1 the measurement of the resistance of transport-deficient mutants to other toxic peptides is one of the stages of characterisation. It would be expected that generally, a mutant defective in *opp* should simultaneously gain resistance to most toxic oligopeptides but still be sensitive to toxic dipeptides. Conversely a *dpp* mutant should retain sensitivity to toxic oligopeptides but gain resistance to a range of dipeptides. The double-permease deficient mutants would be expected to become more resistant than either singly-deficient parent and the triple permease-deficient mutant would be expected to be resistant to all toxic peptides, if there are indeed only three permeases. In addition, if a strain is still sensitive to a dipeptide containing the toxic moiety used in a tripeptide during selection, or vice-versa, then resistance is not due to resistance to the toxic moiety *per se*.

Methods

Agar plate inhibition zone assays were performed as described in section 2.4. Growth studies were performed by

monitoring the A_{660} of a culture in a Bausch and Lomb Spectronic 20 spectrophotometer.

Results

The results of the inhibition zone assays are shown in Table 3.4. The presumptive triple permease deficient-mutant (PA0122) is highly resistant to all the toxic peptides used and is not inhibited even by 2-3mg of solid of these peptides loaded onto the plate (data not shown). The two presumptive double mutants (PA0119 which is dpp opp and PA0113 which is opp dpp) show similar susceptibilities to the peptides used and are both more resistant than either of the two presumptive single permease mutants (PA0107 which is dpp and PA0112 which is opp). Ala-AlaP was found to be still almost as toxic to PA0119 and to PA0113 as to the wild type suggesting that it is transported almost exclusively through the Opt. The presumptive opp mutant (PA0112) gains marked resistance to all the tripeptides tested but is still susceptible to the dipeptides, while the presumptive dpp mutant (PA0107) gains resistance to all the peptides used but less so towards Ala-Ala-aminoxyAla than the dipeptides tested. Growth studies with PA0107 show that it also gains resistance to other dipeptides (see Figure 3.2). PA0107 also gains resistance to triornithine.

Table 3.4 Response of wild type and peptide transport deficient strains of E. coli W to toxic peptides

Peptide	M26-26	PA0107	PA0112		PA0119		PA0122	
	WT	dpp	opp	dpp	opp	opp	dpp	dpp opp opt
Ala-aminoxy-Ala (100)	47	18	45	12		11		0
Ala-Ala-aminoxy Ala (10)	33	30	0	0		0		0
BocPro-Ala-aminoxyAla (100)	44	ND	0	0		0		ND
Ala-Ala-hydraz-inoAla (100)	35	ND	0	0		0		ND
Ala-AlaP (100)	30	25	30	24		25		0
Glu-AlaP (100)	ND	ND	ND	20		19		0
Gly-Gly-Norleu (1mg)	30	26	0	0		0		ND
Orn-Orn-Orn (300)	28	0	0	0		0		0

Amounts of each peptide loaded are given in nmol.

All values are inhibition zone diameters in mm.

ND, not determined.

For structures of peptides see Appendix B.

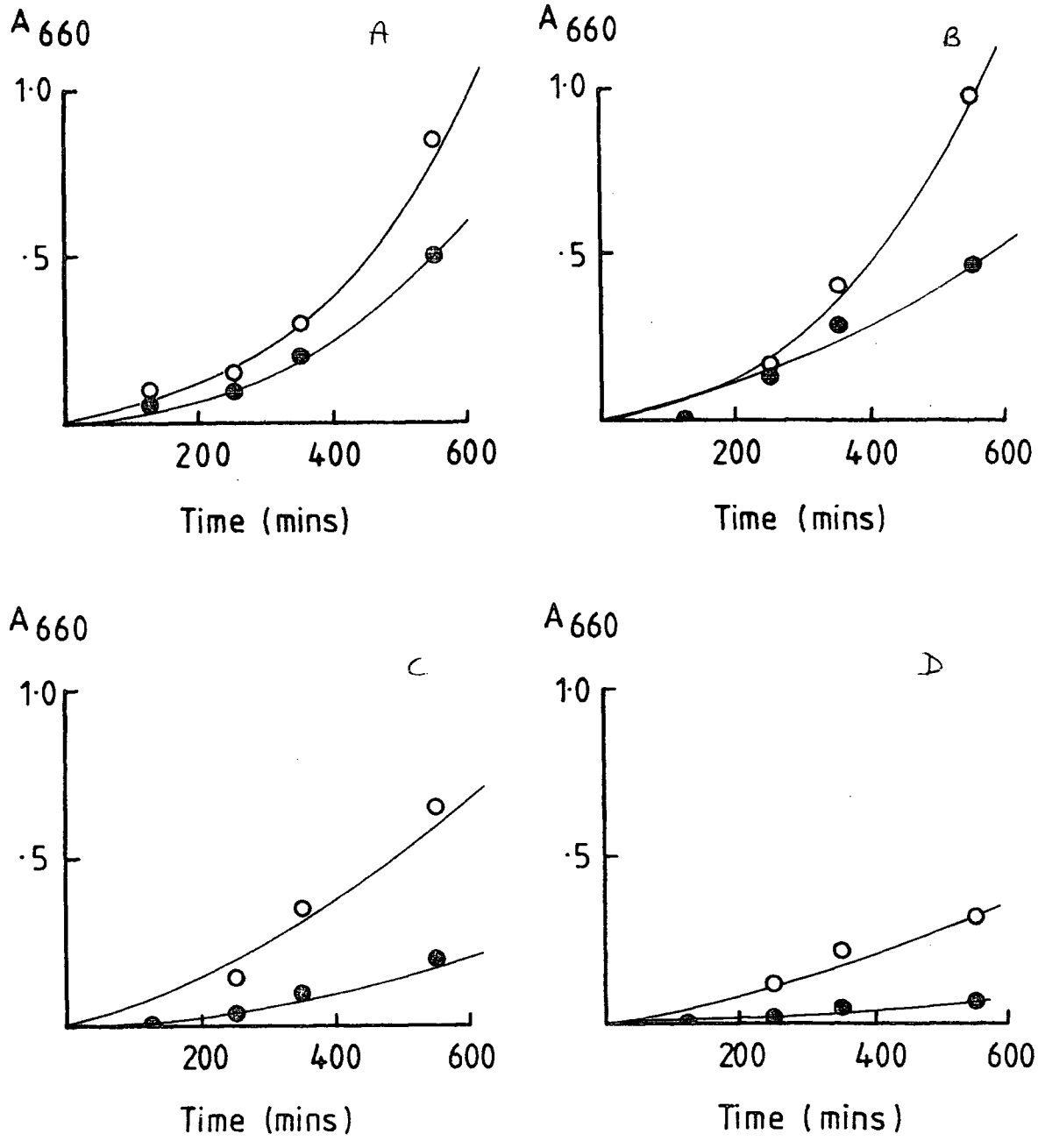


Figure 3.2 Inhibition of growth of wild type (M26-26) and putative dipeptide permease deficient (PA0107) strains by toxic dipeptides. A, glycylnorvaline; B, alanylnorvaline; C, norleucylnorvaline; D, glycylnorleucine. ● M26-26, ○ PA0107.

Discussion

PA0112 (*opp*) remains susceptible to Ala-aminoxyAla while gaining resistance to Ala-Ala-aminoxyAla, whereas PA0107 (*dpp*) remains susceptible to Ala-Ala-aminoxyAla while gaining resistance to Ala-aminoxyAla. It follows that the cause of resistance in both these strains is not resistance to the aminoxy moiety *per se*. The simultaneous gain in resistance to many di- or tripeptides by PA0107 and PA0112 respectively indicates that altered peptidase activity is also probably not responsible for the gain in resistance either. Several different peptidases would have to be affected to account for resistance to the wide range of peptides involved.

The double mutants have similar susceptibilities, indicating that the order of mutation is not important. They are both highly resistant to a range of toxic di- and tripeptides making it unlikely that resistance is due to a change in peptidase activity or resistance to the toxic moiety. Strain PA0122 (the triple deficient mutant) gains resistance to Ala-aminoxyAla, Ala-AlaP and Glu-AlaP simultaneously, again indicating that resistance is not towards the toxic moiety itself and is probably not a change in peptidase activity.

In principle there are several possible explanations for the observation that the presumptive dpp mutant (PA0107) is resistant to triornithine. Triornithine could be transported via both the Opp and Dpp and require a high intracellular concentration to be inhibitory, which cannot be attained without both systems being functional. Alternatively, the Opp and Dpp may share some components so that a single mutation in a shared component could affect both systems. A third possibility is that the presumptive dpp mutant is in fact a double mutant with a defect in opp in addition to a defective dpp. If this third possibility were so then it would be expected that a total loss of Opp activity could then be achieved by another mutation such as that giving rise to PA0119 (dpp opp) which would then be phenotypically identical to the double mutant (PA0113) isolated from an opp mutant. In fact recent genetic evidence (J.W. Payne, personal communication) has shown that PA0107 does contain a mutation in the opp locus as well as the dpp locus. It is clear from the resistance studies that PA0107 does not have a complete loss of Opp function as well, because it is more sensitive to toxic tripeptides than is PA0112, a single opp mutant. The evidence from these subsequent studies of this extra partial mutation in PA0107 should be borne in mind when interpreting the results of the rest of this study.

3.6 PEPTIDE TRANSPORT IN MUTANTS OF E. COLI W RESISTANT TO TOXIC PEPTIDES

Introduction

The evidence presented in section 3.4 makes it unlikely that the resistance to toxic peptides observed in the mutants is due to resistance to the toxic moieties *per se* or to changes in peptidase activity, although stronger evidence regarding the latter is presented in section 3.7. The remaining mechanism to explain the resistance is that peptide transport activity has been lowered in the mutants. Thus, the rates of transport of a range of peptides were tested in the various mutants using the automated fluorescamine assay (Payne and Nisbet, 1981).

Previous studies, mainly using growth tests, have indicated that the *dpp* is specific for dipeptides (Payne, 1968; Payne and Bell, 1979), *opp* transports both di- and oligopeptides (Payne, 1968; Payne and Bell, 1979) and *opt* is restricted to a few tripeptides (Naider and Becker, 1975; Barak and Gilvarg, 1975). Direct measurement of peptide transport in the strains isolated here has provided more detailed information on the specificities of each of the permeases than has been previously available.

Methods

The peptide uptake assays were performed as described in section 2.5.

Results

The results of the uptake assays are shown in Table 3.5. PA0112 (*opp*) shows decreased uptake activity towards all the peptides tested while PA0107 (*dpp*) shows decreased activity towards all the dipeptides tested and most of the tripeptides. Both of the double mutants (PA0119 and PA0113) show less uptake activity than their parent strains and both show similar uptake activity to each other. PA0122 (*dpp opp opt*) has lost uptake activity to all tested peptides, except surprisingly, apparently towards Ala-Lys. Uptake of glutamine, aspartic acid and lysine was also measured in PA0122 and in the wild type (results not shown). The rates were similar in both strains, indicating that the lack of peptide transport activity in PA0122 is not a general lack of active transport but is restricted to peptide transport. The uptake of Gly-[U ^{14}C]Phe was also measured in the wild type, and strains PA0112, PA0107 and PA0119 as a comparison with the fluorescamine results. The ratios of rates of uptake between the strains are similar using both assays but the rates obtained from the ~~fluorescamine~~ radiolabel assay are approximately

Table 3.5 Peptide transport in wild type and permease deficient strains of E.coli W.

	M26-26	PA0112	PA0107	PA0119	PA0113	PA0122
	WT	opp	dpp	dpp opp	opp dpp	dpp opp opt
Ala-Ala-Ala	29	-	19	19	-	ND
Ala-Gly-Gly	34	-	3	4	-	-
Gly-Gly-Gly	26	ND	25	ND	ND	-
Leu-Leu-Leu	25	19	22	17	-	ND
Met-Ala-Ser	50	28	31	7	-	-
Met-Gly-Met	73	33	38	31	31	ND
Val-Val-Val	44	15	16	12	13	ND
Ala-Ala	42	-	35	32	-	ND
Ala-Lys	-	-	-	-	-	24
Gly-D-Leu	ND	-	ND	ND	-	-
Gly-Phe	59	49	29	21	24	ND
Gly-Tyr	40	32	18	15	-	ND
Leu-Leu	-	-	-	-	-	ND
Lys-Lys	28	25	6	1	1	ND
Val-Val	68	-	22	15	-	-
Gly-Gly	28	-	3	ND	ND	-
Gly-[U-^{14}C]Phe	21	17	13	8	-	-

All values in nmol peptide transported min^{-1} mg cellular $protein^{-1}$. All results from the automated fluorescamine assay except Gly-[U- ^{14}C]Phe which is radioactively labelled peptide assay. - not determined.
ND not detectable

40% ~~higher than~~ of those obtained from the ~~radioactively labelled~~ fluorescamine peptide assay.

Discussion

All the mutants lose transport activity towards a range of peptides, providing direct evidence that changes in peptidase activity are not responsible for the effects seen.

The general loss of uptake activity of PA0112 confirms the conclusions of earlier studies (Payne, 1968; Barak and Gilvarg, 1974; 1975; Naider and Becker, 1975), that the Opp can transport a wide range of both di- and oligopeptides.

PA0107 has also lost the ability to transport both di- and tripeptides. The reduction of transport in PA0107 seems to be more marked towards dipeptides than tripeptides. Previous studies of dipeptide transport in E. coli (Payne, 1968; De Felice et al., 1973) indicated that the Dpp was specific for dipeptides. Loss of this system would not be expected to produce a decrease in transport activity towards tripeptides. The presence of an additional mutation in opp in this strain complicates the interpretation of these results as the loss of activity towards tripeptides could be a result of a partially active Opp. Studies using a strain in which an Opp has been transferred into PA0107 by conjugation are

compatible with the idea that the loss of Dpp activity alone causes a decrease in the transport of tripeptides (J.W. Payne, personal communication). Thus, present studies indicate that the Dpp is involved in transporting both di- and tripeptides and has wider specificities than was at first thought.

The similarity of rates of transport in PA0119 and PA0113 corroborates the results of section 3.5 which showed that both double mutants were similarly susceptible to a range of toxic peptides. The double mutants appear to be phenotypically identical, indicating that they both lack Dpp and Opp activity. The uptake seen in the double mutants is likely, therefore, to be a result of the activity of the Opt system. This uptake is abolished by a single further mutation, indicating that there are only three peptide permeases in E. coli W. Earlier growth studies (Naider and Becker, 1975; Barak and Gilvarg, 1975) have shown that tripeptides containing Met, Leu and Thr could be transported via Opt. Based on these studies these authors concluded that the Opt system was specific for these peptides only. The results presented here show clearly that the Opt can transport a wide range of di- and tripeptides and is less specific than was at first thought.

PA0122 still shows apparent uptake of Ala-Lys. Arsenate does not inhibit this apparent uptake (results not shown), which indicates that this is not active transport. The high rate of apparent uptake also indicates that this is not a passive diffusion process. In considering these results it should be remembered that not only does peptide transport cause a decrease in fluorescence but so also will peptide hydrolysis at the standard pH of the automated assay, so that the apparent uptake of Ala-Lys could be a result of the activity of extracellular or periplasmic peptidases. The signal peptidase(s) in E. coli are active in the periplasmic space, or more likely, bound to the outer surface of the cytoplasmic membrane. The sites of cleavage of the signal peptides of many outer membrane and periplasmic proteins have been determined (Perlman and Halvorson, 1983) and they often occur at an Ala-X sequence (where X is another amino acid). Although signal peptidase(s) have not been shown to have dipeptidase activity, the apparent uptake of Ala-Lys could be caused by cleavage by a signal peptidase. Interestingly, it was found earlier by T.M. Nisbet in this laboratory (personal communication) that a peptide transport deficient strain of Streptococcus faecalis also showed apparent uptake of Ala-Lys, perhaps a similar mechanism also applies in this case. It would have been relevant here to test whether using the automated assay at a higher pH would show an increase in fluorescence yield, indicating extracellular cleavage.

The rates of transport obtained using the radioactively labelled peptide assay are slower than those obtained from the fluorescamine assay, as shown previously (Payne and Nisbet, 1980a), because amino acid exodus occurs concomitant with peptide transport in E. coli. A labelled amino acid which has been effluxed cannot be distinguished from untransported peptide in the radioactively labelled peptide assay, whereas amino acid has a much lower fluorescence yield than peptide in the fluorescamine assay, therefore amino acid exodus interferes with the fluorescamine assay much less than it does with the radioactively labelled peptide assay (Payne and Nisbet, 1980a).

3.7 PEPTIDASE ACTIVITIES IN MUTANTS OF E. COLI W RESISTANT TO TOXIC PEPTIDES

Introduction

One of the possible mechanisms described in section 3.1 by which cells can gain resistance to a toxic peptide is a change in peptidase activity. Activity can either be lowered to decrease the rate of release of a toxic moiety from a peptide, or can increase to cleave a peptide more rapidly if intact peptide is required for the toxic effect. Each of the mutants used in this study shows both an increase in

resistance and a loss of transport activity towards a wide range of peptides (see sections 3.5 and 3.6). This indicates that changes in peptidase activity are not responsible for these effects as one would not expect a change in a single peptidase to affect the uptake of so many structurally different peptides. Some form of coordinate control of peptidase activities would be required to show these effects, however no such such control has been shown to occur.

To discount this possibility, the intracellular peptidase activities towards several peptides were directly measured. The peptides used were chosen as those the uptake of which had been markedly reduced in the mutant strains, because any change in peptidase activity would presumably be greatest towards these. Peptidase activity towards the toxic peptides used for selection could not be measured because of the small quantities of these peptides which were available for use at that time.

Aminopeptidase N has been reported to be bound to the outside of the cytoplasmic membrane in E coli K10 and to behave like a cytoplasmic enzyme in all the other strains tested (Murgier et al., 1977). In E. coli K10, this enzyme shows some limited activity mainly towards alanine containing di- and oligopeptides (Chappelet-Tordo et al., 1977) but is mainly active towards amino acid p-nitroanilides and amino

acid B-naphthylamides. It is theoretically possible that aminopeptidase N might be involved in peptide transport by cleaving the peptide and transferring the residues across the membrane, analogous to the mechanisms envisaged by Ugolev (Ugolev et al., 1977). To check this possibility aminopeptidase N activity was measured in wild type and mutant strains, using the increase in absorbance at 410nm caused by release of p-nitroanilide as an assay.

Methods

The intracellular peptidase and aminopeptidase N activities were measured as described in section 2.7.

Results

The peptidase activities measured in the wild type and mutant strains are shown in Table 3.6. The activities of both intracellular peptidases and aminopeptidase N are similar in every strain.

Discussion

The results described in sections 3.3, 3.5 and 3.6 indicate that changes in peptidase activity are unlikely to be involved in the resistance of the mutants to toxic

Table 3.6 Peptidase activities in wild type and peptide transport deficient strains of E. coli W.

Substrate	M26-26	PA0112	PA0107	PA0119
	WT	opp	dpp	dpp opp
Lys-Lys	0.7	-	0.9	0.9
Gly-Tyr	0.9	-	1.5	1.6
Asp-Ala	0.14	-	0.15	0.16
Ala-Gly-Gly	0.5	-	0.5	0.5
Orn-Orn-Orn	ND	-	ND	ND
Alanyl-p-nitroanilide	0.8	0.7	0.6	—

All values are umol amino acid formed min^{-1} mg cellular $protein^{-1}$.

ND not detectable

- not determined

peptides because of the wide range of peptides affected, which would be beyond the specificities of a single peptidase. Since this study was performed, further supplies of the toxic peptides used for selection have become available and the peptidase activities of the wild type and mutant strains towards them have been measured. Peptidase activity towards all peptide analogues was identical in all of the strains tested (J.W. Payne, personal communication). This complements the results presented in this section and shows that changes in peptidase activity are not responsible for the resistance to toxic peptides.

The only mechanism, therefore, which can account for all the effects shown during the characterisation of the mutants is that they are peptide transport deficient.

3.8 EFFECT OF METABOLIC INHIBITORS ON PEPTIDE UPTAKE IN E. COLI W

Introduction

Active transport systems in E. coli are usually either energised by a transmembrane proton gradient the so-called proton motive force (p.m.f.) or by hydrolysis of ATP or some other molecule containing a high energy phosphate bond

(Harold, 1972; Berger, 1973). The particular mode of energisation of a transport system can be determined in several ways, for example: 1) By measuring the effect of a particular osmotic shock treatment on transport, as p.m.f. linked systems are relatively resistant to such treatment whereas phosphate bond-linked systems are sensitive to osmotic shock, probably because osmotic shock releases periplasmic binding proteins from these latter systems (Wilson and Smith, 1978). 2) By studying transport in mutants unable to perform oxidative phosphorylation (unc mutants), using several respiratory substrates and electron donors. 3) Membrane vesicles can be used to reconstitute transport systems and study the effects of different energy sources on transport. Phosphate bond-linked systems are not active in vesicles, as the periplasmic binding proteins are absent. 4) By investigating the effects of metabolic inhibitors, several of which have (relatively) specific effects on one or other of the mechanisms of energisation, for example, protonophores such as carbonylcyanidechlorophenylhydrazone (CCCP) arsenate and N-N´ dicyclohexylcarbodiimide (DCCD). CCCP permeabilises the membrane towards protons and thus destroys the proton gradient. Arsenate is a phosphate antagonist, lowering the ATP levels in the cell (Klein and Boyer, 1972), thus inhibiting phosphate bond coupled transport systems (Cowell, 1974). DCCD is an inhibitor of the membrane bound ATPase which is responsible for maintaining the transmembrane proton

gradient (Evans, 1970) and therefore inhibits transport systems linked to the p.m.f..

Cowell (1974) showed that Gly-Gly transport in E. coli was linked to phosphate bond energy by a combination of the methods described above. Payne and Bell (1979) extended the work of Cowell (1974), confirming that dipeptide transport is phosphate bond linked and demonstrating that the opp is similarly energised. These studies used unc mutants with several energy sources and various metabolic inhibitors. No reports regarding the mode of energisation of the opt system have been published to date.

In this section, the effects of arsenate and DCCD on peptide transport have been studied in the wild type and peptide transport deficient mutants isolated.

Methods

Peptide transport assays were carried out using the automated fluorescamine assay as described in section 2.5. When testing the effects of arsenate, phosphate buffer cannot be used, as phosphate antagonises the effect of arsenate. For experiments using arsenate, cells were resuspended to an A_{660} of 0.2-0.4 in 100mM Tris HCl buffer, pH7.2 and incubations containing arsenate were compared with controls

also in Tris buffer. Both DCCD and arsenate were added to the cells and preincubated at 37°C for 10min before the addition of peptide.

Results

The effects of DCCD and arsenate on Gly-Phe uptake in the wild type and several mutant strains are shown in Table 3.7. 30mM arsenate inhibits uptake of Gly-Phe in all strains by about 60% whereas DCCD has no effect.

Discussion

The differential effect of arsenate and DCCD on the double mutant (PA0119), in which peptide transport activity presumably is due to the opt alone, shows that this transport system is linked directly to high energy phosphate bond hydrolysis. This is the first demonstration of the mode of energy coupling for the opt. The similarity of the results for the other mutants confirms that dpp and opp are also energised by phosphate bond energy as shown by Cowell (1974) and Payne and Bell (1979).

Table 3.7 Effect of metabolic inhibitors on peptide transport in permease deficient mutants of E. coli W.

	M26-26	PA0112	PA0107	PA0119
	WT	dpp	opp	dpp opp
Gly-Phe	59	49	29	21
+ Arsenate (30mM)	24	18	11.5	9.5
% uptake remaining	41	40	37	45
+ DCCD (1mM)	55	49	27	21.5
%uptake remaining	93	100	93	102

Uptake rates shown as nmol peptide transported min^{-1} mg cellular $protein^{-1}$.

3.9 MAPPING OF THE LOCI INVOLVED IN PEPTIDE TRANSPORT IN E. COLI W

Introduction

One of the first stages of bacterial genetic analysis is to locate the position of a gene on the chromosome. The fine structure of the gene can then be analysed using the adjacent genes as reference points. There are several ways of initially locating a gene on the chromosome, but one of the most widely used methods is by F′ donor conjugation, which is the method used here. The principles of this method are described in section 2.8.

The *opp* locus in *E. coli* has been shown to map at 27min (Barak and Gilvarg, 1974; De Felice *et al.*, 1973; Lenny and Margolin, 1980) and the *dpp* between 9 and 27min (De Felice *et al.*, 1973). A report that the *dpp* mapped at 5 min (Bachmann *et al.*, 1976) has been found to be incorrect (Hartmann, 1980). There has been no report on the location of the *opt* locus.

Here the positions of the loci relating to peptide transport have been mapped by selecting for enhanced ability of peptide transport deficient lysine auxotrophs to grow on lysine peptides, when mated with donor strains carrying

functional peptide permease genes on F′ episomes. Knowing the regions of chromosomal DNA carried by each F′ allows the approximate chromosomal location of the complemented gene to be determined.

Methods

The growth of the strains used, the mating procedure followed and the selection of exconjugants are described in section 2.8. The strains used and their genotypes are described in Table 2.1. The F′ episomes carried by the strains are shown in Table 3.8 and the regions of chromosomal DNA which they carry are shown in Figure 3.3.

Results

The donor strains, and the F′ episomes carried, which produced enhanced growth of recipient cells on lysine peptides are shown in Table 3.9. Episome F254 (8-14min), in two different donor strains (KL719 and X573) complemented PA0107 to produce enhanced growth on Lys-Lys. This shows that the *dpp* locus must lie between 8 and 14min on the chromosome, agreeing with the earlier reports of De Felice *et al.* (1973) and Hartmann (1980). The *opp* mutation is complemented by episomes F126 (16-30min) and F123 (27-30min) and therefore maps between 27 and 30min. This position is in

Table 3.8 F′ donor strains and their episomes.

Strain No	Episome	Dist. Cov.	Markers
4251	F104	0-6	thr-argF
4288	F128	6-8	proA-lac
4282	F254	8-14	lac-lip
6350	F254	8-14	lac-lip
4287	F152	14-17	lip-gal
4253	F126	16-30	rac-nadA
4256	F123	27-30	rac-galU
5505	F500	34-44	his-relB
5760	F506	36-37	pps-man
4326	F150	41-44	his-eda
4280	F129	44-50	dsdA-his
4279	F142	51-56	tyr-supN
4291	F143	56-61	lysA-tyrA
4254	F116	60-65	metC-fuc
4248	F141	68-75	asd-argG
4289	F140	68-80	mtl-argG
4258	F111	81-91	pyrE-malB
4265	F133	84-89	ilv-argH
4260	F112	88-98	metB-uxuA
4255	F117	93-96	mel-pyrB

Strain numbers are E. coli Genetic Stock Center numbers. Distances are given in min. Markers are those nearest to each end of the episome.

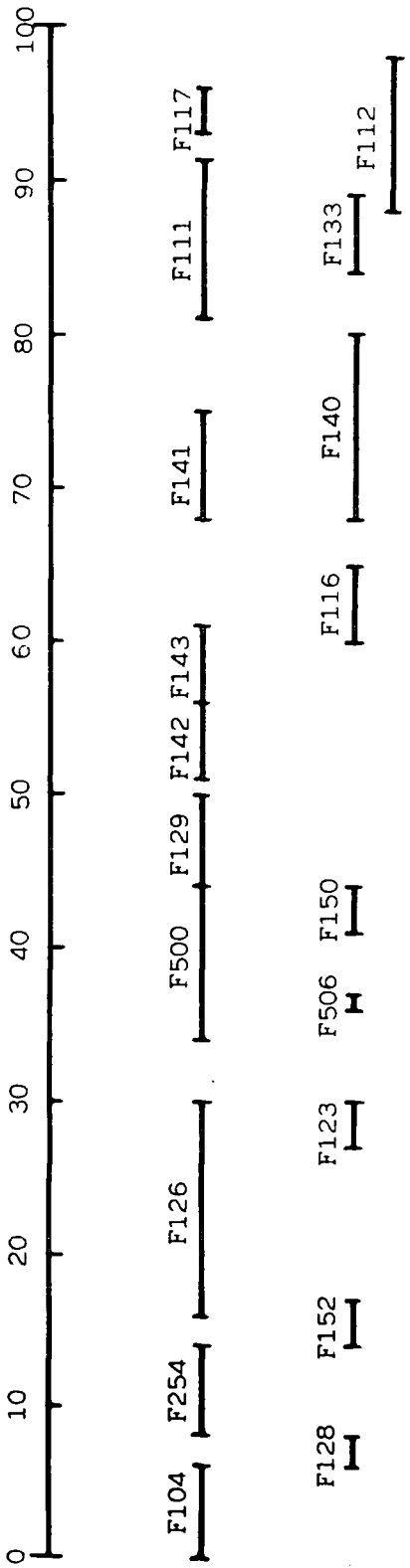

0
10
20
30
40
50
60
70
80
90
100
F104
F254
F128
F126
F152
F123
F500
F129
F506
F150
F142
F143
F116
F141
F140
F111
F133
F117
F112

Table 3.9 Complementation of permease deficiency by F′ episomes.

Strain No.	Episome No.	Markers	Min.	Permease
KL719	F254	lac-lip	8-14	dpp
X573	F254	lac-lip	8-14	dpp
KL703	F126	rac-nadA	16-30	opp
KL701	F123	rac-galU	27-30	opp
KL728	F111	pyrE-malB	81-91	opt
KL706	F133	ilv-argH	84-89	opt

Permease is the mutant permease in the recipient which is complemented by the F′ episome mated into it.

agreement with the earlier reports that opp maps at 27min (Barak and Gilvarg, 1974; De Felice et al., 1973; Lenny and Margolin, 1980). The opt mutation in the triple mutant (PA0122) was complemented by episomes F111 (81-91min) and F133 (84-94min) but not by F112 (88-98min). The opt locus must therefore lie between 84 and 88min on the chromosome. The restoration of opt activity in cells from the PA0122 crosses with F111 and F133 were confirmed by isolation of lysine auxotrophic exconjugants which had regained sensitivity to Ala-AlaP (the toxic peptide used to select for an opt deficient mutant in section 3.3). Crosses between PA0122 and F112 did not produce exconjugants which had regained sensitivity to Ala-AlaP. Episome F143 produced enhanced growth of all strains on all lysine sources as it contains the lysA gene and thus complements the mutation which produces lysine auxotrophy in the recipient cells.

Discussion

Because of the overlapping specificities of the three peptide transport systems, dpp or opp strains still grow on Lys-Lys or Lys-Lys-Lys respectively as lysine donors. When a transport system is restored after conjugation the growth rate on lysine peptides is enhanced sufficiently for faster growing colonies to be detectable after a few hours incubaton as described in section 2.8. The selection is not ideal

because of this background growth of original mutant, therefore the position of the opt was confirmed by testing exconjugant cells for restoration of Ala-AlaP sensitivity. More recent work (J.W. Payne, personal communication), measuring peptide uptake in exconjugants of the triple mutant and F111 and F133 crosses, has shown that they have similar transport characteristics to the double permease deficient mutants used in this study. This is further evidence that opt activity has ben restored by these crosses and that the opt locus lies between 84 and 88min.

The presumptive dpp mutant (PA0107) only showed complementation with episomes containing the region with the dpp locus. Further studies measuring peptide uptake in exconjugants (J.W. Payne, personal communication), have shown that this mutant also contains a mutation in the opp locus. This mutation was not detected in this study, probably because the increase in growth rate produced by restoration of a fully active opp system from a partially active one was not sufficient to be measured by the selection procedure used here.

The two double peptide permease deficient mutants (PA0119 and PA0113) showed enhanced growth on Lys-Lys or Lys-Lys-Lys when crossed with donor strains carrying episomes with either the dpp or opp loci respectively, as might be expected. They

provide confirmation of the results obtained with the dpp and opp single mutants.

The ability of episome F143 to complement the lysA mutation in all the recipient strains provides a useful control, demonstrating the ability of each recipient strain to mate, and giving an estimate of the frequency of conjugation. As discussed in section 2.8, the frequency of conjugation is low, but this would be expected between two different strains of E. coli (the donor strains are E.coli K12, while the recipient strains are E. coli W).

3.10 THE EFFECT OF ALA-LYS ON ALKALINE PHOSPHATASE PRODUCTION IN E. COLI W

Introduction

The triple peptide permease deficient mutant (PA0122) was shown in section 3.6 to be totally defective in peptide transport, except apparently for some activity towards Ala-Lys. This apparent uptake was not inhibited by arsenate and is therefore probably not active transport. One possibility is that the Ala-Lys is being cleaved by a periplasmic peptidase. This would cause a decrease in the fluorescence yield measured by the fluorescamine assay and

would appear to be peptide uptake. The signal peptidases possess endopeptidase activity towards the leader regions of nascent polypeptide chains and have been found associated with both inner and outer membrane fractions of E. coli (Zwizinski et al., 1981). It is interesting to note that many leader regions are cleaved on the carboxyl side of an alanine residue and in the case of the maltose binding protein, cleavage occurs between alanyl and lysyl residues (Osborn and Wu, 1980). Presumably the signal peptidase should be accessible to Ala-Lys in the periplasmic space and might possess some limited activity towards this peptide. If signal peptidase activity is involved in this apparent uptake, then Ala-Lys should be able to compete with leader polypeptide chains for the peptidase active site. A high concentration of Ala-Lys might therefore interfere with the rate of production of a periplasmic or outer membrane protein by competing for cleavage of the pre-protein. Alkaline phosphatase lends itself to this type of study in several ways. It is a periplasmic enzyme, and therefore has to be processed by a signal peptidase before the mature protein can be released. Its presence is easily monitored by following the production of p-nitrophenol (which absorbs at 415nm) from p-nitrophenylphosphate. The enzyme is inducible under conditions of low phosphate, so that if cells are transferred from a high phosphate to a low phosphate buffer at the start of the assay, there is little baseline activity and most of

the activity detected is newly synthesised protein. It was therefore decided to follow the production of alkaline phosphatase in the presence or absence of high concentrations of Ala-Lys.

Methods

Alkaline phosphatase production was followed in PA0122 as described in section 2.10 in the presence of 50mM Ala-Lys or 50mM Ala-Ala as a control. Cells without substrate and uninduced cells were also used as further controls.

Results

The production of alkaline phosphatase, under the incubation conditions described above, is shown in Figure 3.4. Very little activity is seen in cells incubated with 100mM PO_4 (uninduced) and only a slight increase in absorbance, probably caused by cell growth, is seen in the absence of substrate. Induced cells in the presence of Ala-Ala or Ala-Lys produce alkaline phosphatase activity. Slightly more activity is produced in the presence of Ala-Ala than Ala-Lys.

Discussion

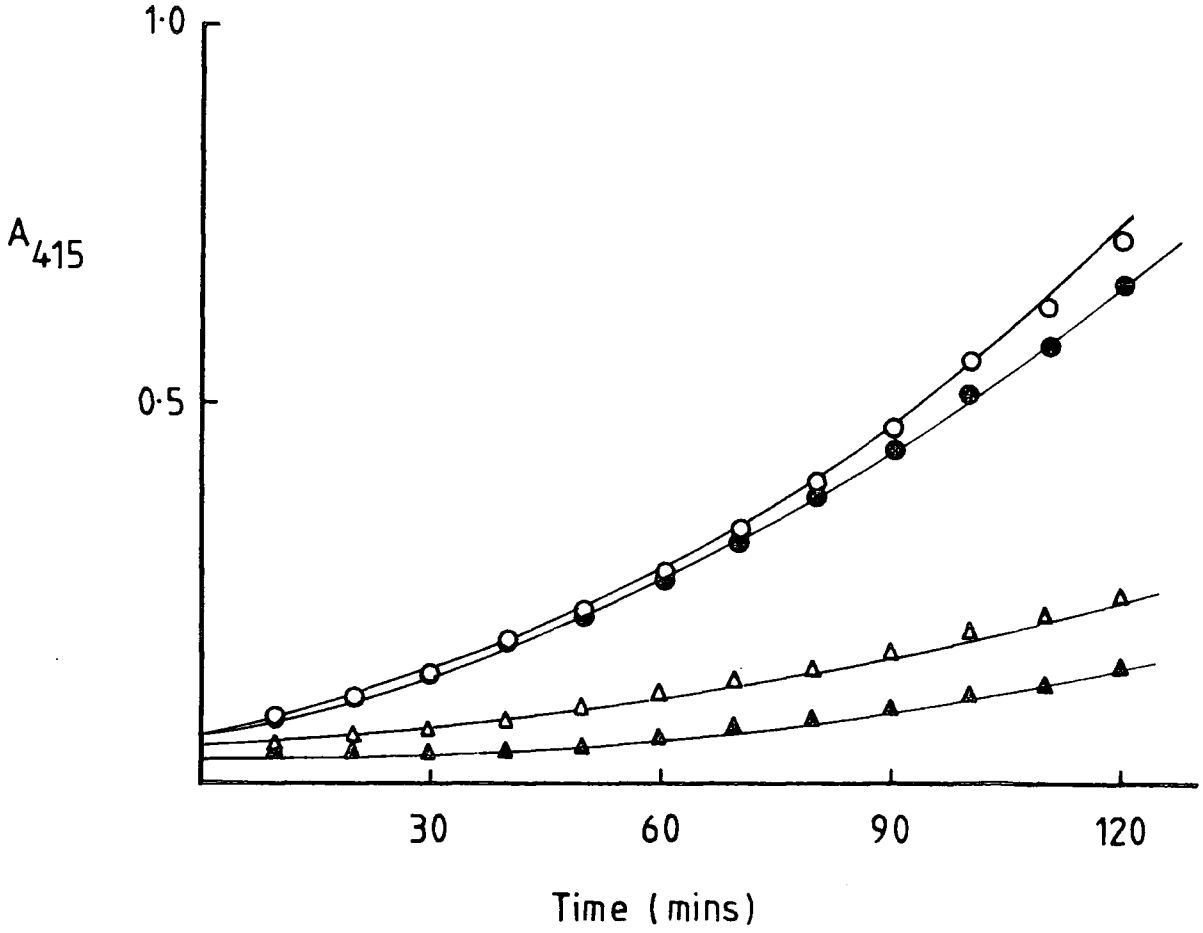


Figure 3.4 Effect of Ala-Lys on alkaline phosphatase production by PAO122.

▲, No substrate; △, uninduced cells with substrate; ○, induced cells with substrate and 50mM Ala-Ala; ●, induced cells with substrate and 50mM Ala-Lys.

Uninduced cells or cells without substrate, produce very little increase in A_{415} as would be expected, indicating that little alkaline phosphatase is produced in uninduced cells and that there is no production of any other substance which absorbs at 415nm in the absence of substrate. Production of alkaline phosphatase is slightly lower in the presence of Ala-Lys than in the presence of Ala-Ala. This could occur by several mechanisms; 1) there could be fewer cells in the Ala-Lys incubation; 2) Ala-Ala might be providing an extra nitrogen source for the cells, thus allowing more rapid protein synthesis than in the presence of Ala-Lys; 3) Ala-Lys could be competing with alkaline phosphatase for the active site of the signal peptidase, whereas Ala-Ala was not. 1) is unlikely as cells were aliquots of the same suspension and all other volumes added were identical. 2) is also unlikely as Ala-Ala is not detectably transported into PA0122, whereas if Ala-Lys is being cleaved to alanine and lysine, these can then be taken up via the amino acid permeases and be used as nitrogen sources. It is possible therefore that this slight inhibition of alkaline phosphatase production detected is a result of competition between Ala-Lys and the nascent polypeptide chain for the active site of the signal peptidase. It should be stressed that the levels of peptide used here (50mM) are much higher than those used in normal transport studies and that the minimal effect shown by Ala-Lys even at this concentration indicates that there must

be little if any affinity between Ala-Lys and the signal peptidase. The inhibition of alkaline phosphatase is likely to be an underestimate as Inouye and Beckwith (1977) have reported that the pre-alkaline phosphatase protein with the signal peptide intact still possesses some phosphatase activity. Inhibition of signal peptidase activity might therefore, not prevent production of alkaline phosphatase but produce a molecule with lower activity.

3.11 CONCLUDING DISCUSSION

The results presented in this chapter extend our knowledge of the number and specificities of the peptide transport systems in E. coli. Naider and Becker (1975) suggested that there might be several peptide transport systems in addition to the Opp and Dpp systems. Clearly, there are only three peptide transport systems in E. coli, the Opp, Dpp and Opt. Earlier studies (Naider and Becker, 1975; Barak and Gilvarg, 1975) using growth assays, were interpreted as indicating that transport via the Opt system was limited to a few tripeptides containing Met, Leu and Thr. The results obtained here, using the direct fluorescamine assay show that, although Leu-Leu-Leu and Met-Gly-Met are largely transported via the Opt system (confirming the earlier

reports), the Opt also transports a wide range of other peptides. This emphasises the advantages of direct asays over indirect growth assays. The opt locus is shown here to lie between 84 and 88min on the E. coli chromosome. This is the first report of the location of opt . It should facilitate the analysis of the fine structure of the gene(s) involved. The Opt system has also been shown to be energised by phosphate bond energy, like the Dpp and Opp systems.

Studies of peptide transport in PA0107 (dpp) suggest that the Dpp is somehow involved in the transport of both di- and oligopeptides. This is an extension of the earlier reports of the specificities of the Dpp, which were most easily interpreted as indicating it to be unable to transport oligopeptides. However, the discovery of an additional opp mutation in PA0107 (J.W. Payne, personal communication) confuses this interpretation and makes it difficult to assess the relative contributions of the Opp and Dpp towards overall oligopeptide transport from studies with only the mutants isolated in this study. A strain with an Opp system transferred into it by F′ donor conjugation, but retaining the dpp mutation, still shows decreased transport activity towards tripeptides (J.W. Payne, personal communication). Further studies with this strain should determine the contribution of the Dpp system towards overall oligopeptide transport. The transport of tripeptides indicates that the

Dpp is able to recognise a greater variation in peptide backbone length than was originally thought. The report that the Dpp is also active towards hydrazino and aminoxy peptide analogues (Morley *et al.*, 1983), provides further evidence for its ability to recognise extended peptide backbones. The map location of the *dpp* locus has been narrowed down from 9-27min (De Felice *et al.*, 1973) to 8-14min. Combining the two results narrows the range still further to between 9 and 14min.

The information regarding the specificities of the peptide permeases and the frequency of their spontaneous loss, should help in the rational design of effective peptide carriers and peptide analogue antibiotics. A peptide which is transported more or less equally by two or all of the peptide permeases is likely to be a much better candidate, than a peptide which is largely transported via one permease. The lower mutation frequencies of Dpp and Opt make them potentially more useful systems than Opp, so a peptide antibiotic should be designed to utilise Dpp and Opt rather than Opp. Although it should be noted of course, that the high frequency of *opp* mutation has only been shown for *E. coli* and may not be so for other clinically important bacteria in which Opp is the most effective peptide permease. Originally, it was thought that only the Opp system would have specificities broad enough to transport peptide analogues, but ironically alafosfalin

(Ala-AlaP), one of the first synthetic linear peptide antibacterial agents, is transported almost entirely via the Dpp and Opt.

The study of peptide transport is now well characterised at the physiological level. There has been little work on the molecular mechanisms of peptide uptake and consequently they are poorly understood. The powerful techniques of genetic manipulation now available makes this a potentially exciting and fruitful area of study. The opp locus has been shown to consist of four genes transcribed as a single operon (Hogarth and Higgins, 1983). Other phosphate bond linked systems have also been shown to consist of several genes linked in one operon (Higgins et al., 1983; Ames and Higgins, 1983), with one gene coding for the periplasmic binding protein and others coding for membrane bound proteins. Cloning and sequencing of the loci coding for the Opp, Dpp and Opt systems will enable any areas of homology between the systems to be determined. The sequence data can also be compared with those for other phosphate bond linked transport systems and may provide evidence for certain important sequences which are conserved in different systems and other variable sequences which could be involved in determining the specificities of each system. It should also be possible to determine the cellular location of the permease proteins and their positions on or in the cell membrane. Our

understanding of the molecular processes of peptide transport should, hopefully, be greatly improved over the next few years.

4 THE EFFECT OF OUTER MEMBRANE PROTEIN DEFICIENCY ON PEPTIDE TRANSPORT

4.1 INTRODUCTION

The outer membrane of Gram-negative bacteria forms a highly effective barrier to hydrophobic molecules e.g., Actinomycin D and phenol, but allows the diffusion of small, hydrophilic, nutrient molecules. This diffusion has been shown to occur via non-specific aqueous pores formed by "porin" proteins (see section 1.2). Several classes of hydrophilic molecules (e.g., sugars, nucleotides, amino acids), have been shown to use the porin channels whereas certain other outer membrane proteins e.g., lamB, tsx have been demonstrated to form more specific pores. This study was undertaken to determine which if any of the proteins plays a role in facilitating peptide diffusion across the outer membrane.

Several different approaches have been used in the study of outer membrane permeability. Decad and Nikaido (1976) measured the equilibrium distribution of oligosaccharides between the external solution and the periplasmic space in plasmolysed cells. This technique is simple and rapid but

its usefulness is limited by the need for the use of non-metabolisable molecules, precluding its application to studies on the permeation of nutrient molecules. These experiments show whether or not a molecule can penetrate to the periplasmic space but do not have the necessary precision to allow the kinetics of the process to be determined.

A second approach is to prepare liposomes (phospholipid membrane vesicles) in which outer membrane proteins have been incorporated (Nakae, 1976a,b) and to study their permeability properties in a medium containing both the substrate of interest, plus a control impermeant molecule, that are differentially radioactively labelled. Some of the medium is trapped inside the liposomes as they form so the substrate and impermeant control molecules are present in the same ratio both inside and outside the liposomes. When the external medium is separated from the liposomes by gel filtration the substrate molecules, if permeable, will diffuse out of the liposomes, altering the ratio of impermeant control molecule to substrate molecule. The ratio of labels remaining in the liposomes after filtration can be compared with the ratio in the initial medium to determine whether or not the substrate molecules have been able to diffuse out. Alternatively the liposome swelling assay has been used (Nikaido and Rosenberg, 1983). Liposomes in which porins have been incorporated are prepared in a medium

containing impermeant molecules. After washing the external medium away, the liposomes are resuspended in an isotonic solution of the test molecule. An influx of the test molecule induces an accompanying influx of water which causes the liposomes to swell, altering the optical density of the suspension measured at 440nm. Using these reconstituted liposomes allows individual protein species to be tested and also allows metabolisable molecules to be used. As with the equilibrium studies, the data produced from the radioactive label diffusion method are not precise enough to be used to determine kinetic parameters, and although the liposome swelling assay is more precise it is prone to interference from any small ions present in the external solution, which affect the rate of diffusion into the liposomes. The pore-forming ability of some proteins may however be dependent on, or altered by, interactions with other membrane components, and this situation may be difficult to reproduce using this system so that results obtained are not necessarily indicative of the situation in vivo. Indeed Nikaido and Rosenberg (1983) noted that although measuring rates of permeation through porins in liposomes and intact cells give qualitatively similar results, the differences in rates between differently sized and charged B-lactam molecules were greater when measured in intact cells than when measured in liposomes. This suggests that the pores are more discriminatory in vivo than when present in isolation in

vitro.

Another method used is to measure diffusion rates across the outer membrane in intact cells under conditions in which the substrate is continuously removed from the periplasmic space, maintaining a concentration gradient across the outer membrane. This can be achieved in several ways, e.g., by the rapid breakdown of the molecule in the periplasmic space, as in the studies of Zimmermann and Rosselet (1977) and Nikaido et. al. (1983) who used B-lactam antibiotics and periplasmic B-lactamases, or by the active transport of the molecule across the cytoplasmic membrane. In the latter approach the overall transport kinetics are measured and this approach has been used by Von Meyenburg (1971) and by Bavoil et al. (1977) who measured the influence of porin deficiency on the apparent Km of transport of a variety of substrates e.g., sugars and amino acids. These studies used cellular growth rates as an indirect measure of substrate transport, with the apparent Km of transport being calculated as the substrate concentration at which growth occurs at half the maximal rate. Measurement of growth rates is not an ideal assay as growth is not solely dependent on substrate transport. Bavoil et al., (1977) calculated changes in the value of the outer membrane permeability coefficient (p) based on the growth rate kinetics of porin deficient strains although such estimates must be regarded as highly speculative in view of

the indirect nature of the assay used. A better approach is to measure substrate transport or hyrolysis directly, either by monitoring intracellular accumulation of substrate or its disappearance from the external medium. This approach has been used by Zimmermann and Rosselet (1977) and more recently by Nikaido et. al. (1983) using the hydrolysis of B-lactams by periplasmic B-lactamases. Zimmermann and Rosselet used a microiodometric assay to measure the rate of hydrolysis, but this assay is time consuming and can only provide a few rate measurements during each assay. The hydrolysis of B-lactam molecules causes a change in absorption at 260nm because of the cleavage of the B-lactam ring and this property was used by Nikaido et. al. to follow hydrolysis. The light scattering of whole cells makes the background absorbance very high in this assay, but this was overcome by using a narrow path length (1mm) cuvette and high B-lactam concentrations (1mM). The requirement for high concentrations precludes the use of neutral B-lactams which have low solubilities and reduces the scope of the assay.

In the studies described here the disappearance of peptides from the external medium has been used as a direct measurement of peptide transport. The direct measurement of diffusion kinetics in mutant strains lacking particular outer membrane proteins should, in principle, allow differences in diffusion rates across the outer membrane to be related to

pore type. By correlating the differences in diffusion rate to the size and charge etc., of the substrates used, the specificities of each pore type can be explored.

The recent development of the continuous fluorescamine assay for peptide transport (Payne and Nisbet, 1981) has made it possible to study the permeability of peptides across the outer membrane by using the transport kinetics approach. Peptides make useful substrates for this type of study because they occur in a range of sizes, charges and hydrophobicities etc. so that the effects on diffusion of variations in several parameters can be investigated.

The possible involvement of various outer membrane proteins in facilitating peptide diffusion has been investigated in this study by directly measuring peptide transport in wild type and outer membrane protein-deficient strains. Of the strains tested only those deficient in porins or OmpA had any significant effect on peptide transport.

4.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains and growth conditions used are fully described in section 2.2. For convenience the strains and

their relevant genotypes are also shown in table 4.1. A high level of nutrients is required to minimise selection pressure against mutations such as ompB, which severely reduce the ability of the cells to absorb nutrients. All strains were therefore routinely grown in L-broth. Because of the use of a complex medium which might interfere with the subsequent transport assay, the cells were washed with 2 x 30ml potassium phosphate buffer pH7.2 to ensure complete removal of all growth medium before resuspension. The cells were otherwise treated as in the standard harvesting procedure (section 2.3).

4.3 JUSTIFICATION FOR THE APPLICATION OF MICHAELIS MENTEN KINETICS.

Peptide uptake can be considered to be essentially a 2 stage process: 1) diffusion of peptide across the outer membrane and 2) active transport of peptide across the inner membrane. The object of this study was to determine whether the porins, or any other outer membrane proteins, are involved in facilitating the diffusion of peptides across the outer membrane. If any of these outer membrane proteins are involved then the ease with which peptides diffuse across the outer membrane should be decreased in mutants lacking these proteins.

Table 4.1 Strains used and their outer membrane deficiencies

Strain	Lab No.	OMP deficiency
P400	H1	Wild Type
P400:6	H2	ompC
P400:6hIr	H3	ompC ompA
P530	H4	ompB
CM6	NC1	Wild Type
CM7	NC2	ompB
AB2847	NC3	Wild Type
T19	NC4	ompB tsx
AB2847	B1	Wild Type
P8	B2	tonA
BR158	B3	tonB
RK3931	B4	tonA
WA28	B5	fecB
WA380	B6	fecA
W3110	B7	Wild Type
KB419	B8	lamB
KB423	B9	tsx
JE5512	B10	Wild Type

JE5513	B11	lpp
JF568	568	Wild Type
JF694	694	ompC ompF nmpA1
JF699	699	ompA
JF700	700	ompA ompC
JF701	701	ompC
JF703	703	ompF
SH5014	NS1	Wild Type
SH5551	NS2	ompD
SH6017	NS3	ompC
SH6260	NS4	ompC ompD
HN407	NS5	ompC ompD ompF+++

All strains E. coli K12 except NC1 and NC2 (E. coliB/r) and NS1-5 (S. typhimurium)

Strain AB2847 was supplied as the wild type strain for two different sets of mutants so the strain supplied with each set was used as a reference for those particular strains.

After initial addition of peptide to the external medium, diffusion across the outer membrane is at its most rapid because no peptide is present in the periplasmic space at time 0 and the peptide concentration gradient between the medium and periplasmic space is at its greatest. The rate of active transport across the inner membrane depends on the peptide concentration in the periplasmic space. As the concentration in the periplasmic space rises from continued diffusion across the outer membrane, the rate of active transport also rises. Concomitant with this rise in transport rate is a lowering of the diffusion rate across the outer membrane as the concentration gradient between the external medium and periplasmic space falls. Eventually (probably within seconds of peptide addition) a steady state is reached in which the rate of diffusion across the outer membrane is balanced by the rate of active transport across the inner membrane. The peptide concentration in the periplasmic space will not be the same as in the external medium but will depend on the ease of diffusion across the outer membrane and the rate of removal by the transport system.

Assuming that the peptide transport systems are not directly affected by the outer membrane protein mutations, then the periplasmic peptide concentration will be controlled by the ease of diffusion of peptide across the outer

membrane, which in turn will determine the rate of transport across the inner membrane. Zimmermann and Rosselet (1977) described the relationship between the diffusion and active transport steps and it is this approach that is used as the basis for this study.

The active peptide transport across the inner membrane is analogous to an enzymic process and can in the simplest analysis be considered to conform to Michaelis Menten kinetics :-

$V = VmaxS/Km + S$.....(1)

where Vmax is the maximal transport velocity, Km is the substrate concentration when the velocity is half maximal, V is the transport velocity and S is the substrate (peptide) concentration. The diffusion across the outer membrane can be considered as essentially a physical process to which Fick's law can be applied :-

$V = PA(So-Sp)$.....(2)

where V = rate of diffusion, So and Sp are the peptide concentrations outside in the medium and in the periplasmic space, respectively, P is the diffusion constant and A is the area of the membrane.

At steady state, the rate of peptide diffusion across the outer membrane equals the rate of peptide transport across the inner membrane, therefore, S can be eliminated from

equation (1) and Sp from equation (2) :-

$V = (PASo-V)Vmax/PAKm + PASo-V$.....(3)

Solving for V equation (3) becomes :-

$V=1/2\{Vmax+PAKm+PASo-[(Vmax+PAKm+PASo)2-4SoPAVmax]1/2\}$...(4)

Equation (4) can be rewritten :-

$$\frac{1}{V} = \frac{PAKm}{(PASo-V)} + \frac{(PASo-V)}{Vmax} = \frac{1}{Vmax} + \frac{KmPA}{Vmax\,(PASo-V)} \quad .....(5)$$

From equation (2) PASo-V = PASp

If the permeability of the outer membrane is high So>> Sp and PASo-V>> PASo thus equation (5) becomes :-

$$\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax.So} \quad .....(6)$$

This is the Lineweaver Burke form of the Michaelis Menten equation (equation 1). If the outer membrane permeability is very low then the external peptide concentration must be much higher than the Km to achieve half maximal rates. So > Km and PASo > PAKm, therefore PAKm can be neglected in equation 4.

$V = 1/2\{Vmax + PASo - [(Vmax + PASo)2 - 4SoPAVmax]1/2\}$

$= 1/2\{Vmax + PASo - [Vmax2 - 2PASoVmax + (PASo)2]1/2\}$

$= PASo$.....(7)

Equation 2 predicts that if So is sufficiently high, V will also be high regardless of the permeability of the outer membrane and therefore the Vmax of peptide transport will remain unaffectd by changes in membrane permeability. These calculations also predict that peptide transport rates

measured in strains with a high outer membrane permeability will fit the Michaelis Menten hyperbola, but as membrane permeability decreases then the data produced will tend towards a straight line except at high external peptide concentrations where the resistance to diffusion may be overcome by a large concentration gradient and the active transport system again becomes the limiting factor. At these high external concentrations the relationship between V and So will again conform to the Michaelis Menten equation.

The difference in outer membrane permeability coefficient between the wild type and double porin deficient strains used in this study is unlikely to exceed 20-50 times (Nikaido, 1979) and in this relatively narrow range the application of Michaelis Menten kinetics will provide a useful means of identifying any changes in outer membrane permeability to peptides. The values obtained for Vmax and apparent Km will, however, become less reliable as the outer membrane permeability decreases.

4.4 INITIAL SCREENING OF MUTANTS

Nikaido (1979) warned that uptake assays performed at a single arbitrary substrate concentration would not provide

much useful information about outer membrane permeability. However, an uptake assay performed at a carefully chosen substrate concentration near the apparent Km of the uptake system being used should reveal a decrease in outer membrane permeability as a decrease in transport rate. An assay at a single concentration if successful, would be useful as a method for rapidly screening many outer membrane protein-deficient mutants. All the mutant strains available were assayed for peptide transport at a single concentration to determine the efficacy of such an approach. Particular mutants showing a decrease in peptide transport activity were further tested by kinetic analysis of uptake to extend the results of the initial screen.

Uptake assays were performed as described in section 2.5. using a standard initial peptide concentration of 100uM. Because uptake cannot be measured for 2 minutes after the start of an assay, rates were actually being measured in the concentration range 0-90uM peptide. The typical apparent Km reported for peptide transport (usually Gly-Gly or Gly-Gly-Gly) is in the range 1-50uM (Payne and Bell, 1979; Nisbet, 1980) and usually towards the lower end of this range. Recent results from the continuous fluorescamine assay (J.W. Payne, personal communication) show that the Km for Leu-Leu uptake is 4.5uM so that the peptide concentration used in this initial screen is only slightly above the

apparent Km. Any change in outer membrane permeability which increased the Km of peptide transport would thus be reflected in a decrease in the rate of transport.

The results of this initial screening are shown in Table 4.2. It can be seen from these results that the loss of OmpC does not lead to a reduction of peptide transport, however the further loss of OmpA, or the loss of both porins in ompB strains causes a marked reduction in rates of uptake. None of the other mutants tested shows a decrease in peptide uptake. The T6 phage receptor protein (coded for by the tsx gene) which was originally thought to form a specific pore for nucleoside uptake (Hantke, 1976) has been shown to allow uptake of some amino acids (Heuzenroeder and Reeves, 1981). The specificities of this pore are not yet clear as serine, glycine and phenylalanine used the pore while arginine and glucose did not. The strains used by Heuzenroeder and Reeves overproduced the T6 receptor protein thus the effect of losing it would be greater than in a strain producing a more normal level of protein. There is no evidence to date, including the present study (where there is no decrease in peptide uptake when tsx is lost in strain B9 compared with the parent strain B7), which suggests that the pore formed by the T6 receptor protein plays a significant role in peptide uptake. The phage lambda receptor protein (LamB) forms a pore which is specific for maltose uptake unless the

Table 4.2 Initial screening of peptide uptake in outer membrane protein-deficient strains.

Strain	Lab No.	Genotype	Ala3	Ala2	MGM	G-P	A-P
P400	H1	Wild Type	42	110	70		
P400:6	H2	ompC	49	118	68		
P400:6hIr	H3	ompC ompA	29	64	25		
P530	H4	ompB	14	49	ND		
CM6	NC1	Wild Type	63	52	41		
CM7	NC2	ompB	41	44	17		
AB2847	NC3	Wild Type	27	35	21		
T19	NC4	ompB tsx	4	28	16		
AB2847	B1	Wild Type	31	29	21		
P8	B2	tonA	42	33	29		
BR158	B3	tonB	25	37	28		
RK3931	B4	tonA	28	27	27		
WA28	B5	fecB	70	55	55		
WA380	B6	fecA	30	23	19		
W3110	B7	Wild Type	70	52	52		
KB419	B8	lamB	65	52	40		
KB423	B9	tsx	62	70	55		
JE5512	B10	Wild Type	ND	9	4		

JE5513	B11	lpp	130	180	116		
SH5014	NS1	Wild Type	60	61	30	43	31
SH5551	NS2	ompD	50	48	17	10	15
SH6017	NS3	ompC	48	53	24	39	21
SH6260	NS4	ompC ompD	49	45	17	24	12
HN407	NS5	ompC ompD ompF+++	61	60	44	39	29

All strains E. coli K12 except NC1 and NC2 (E. coliB/r) and NS1-5 (S. typhimurium)

ND not detectable

Transport rates given in nmol min^{-1} mg cellular $protein^{-1}$

Strain AB2847 was supplied as the wild type strain for two different sets of mutants so the strain supplied with each set was used as a reference for those particular strains.

Ala2, Ala3, MGM, G-P and A-P are Ala-Ala, Ala-Ala-Ala, Met-Gly-Met, Gly-Phe and Ala-Pro respectively.

periplasmic maltose binding protein is absent, when the pore takes on the properties of a general diffusion pore (Heuzenroeder and Reeves, 1980). The binding protein was present in the strains used in this study (B7 and B8) so that although no effect on peptide transport was detected, the pore would not have been expected to be acting non-specifically. It would be of interest to compare the apparent rates of peptide transport in isogenic strains with both the LamB protein and the periplasmic binding protein present or absent as some effect on transport might be expected.

B5 (fecB) and B11 (lpp) show an apparent increase in transport activity relative to B1 and B10, their respective wild types. B10 may be a spontaneous peptide transport deficient mutant as it exhibits anomalously low transport activity, and this makes it difficult to assess the effects of mutations in strains derived from it because it is not known whether the transport deficiency was acquired before or after B11 was isolated from it. Such spontaneous transport deficient mutants occur frequently in stock cultures (Payne, 1980) although it is not clear why. However B11 displays an apparent transport activity which is greater than any other wild type strain tested which suggests that the loss of lipoprotein is having some effect which is not directly involved in peptide transport per se. Arsenate was found to

have little inhibitory effect on this apparent uptake (results not shown), which also suggests that peptide transport is not responsible for this activity. B11 completely lacks the lipoprotein which is important in maintaining the structural integrity of the outer membrane (Hirota et al., 1977; Inouye, 1979). This mutant has a "leaky" outer membrane, perhaps allowing peptidases to escape into the external medium. If cleavage of peptide were occurring through release of peptidases the fluorescence yield of samples of the external medium would decrease because under the conditions used in the fluorescamine assay amino acids have a much lower fluorescence yield than isomolar peptides (see section 1.4), this situation would produce similar results mimicking peptide transport activity. Because of the very high level of apparent uptake in B11 it seems likely that at least some of the decrease in fluorescent yield is due to peptidase activity. The fecB gene is involved in the citrate-dependent iron uptake system (Woodrow et. al., 1978). This uptake system is induced by citrate when an 81K protein appears in the outer membrane (Hancock et. al., 1976). Under the conditions used, fecB would not be expected to be induced, so that its loss should not have any effect unless it was still expressed at a low level even when uninduced and had another as yet unrecognised cellular function. The further elucidation of the role of fecB may clarify the position. The use of another fecB

strain would show whether this apparent increase in the peptide diffusion rate was a property of the fecB mutation or a peculiarity of strain B5.

Salmonella typhimurium has three porin types, two of which correspond to the OmpC and OmpF porins in E. coli (Nakae and Ishii, 1978). In E. coli, the loss of one porin type has been shown to lead to an increase in the other porin type (Lugtenberg et.al., 1976), although the exact degree of increase is not yet clear. Heller and Wilson (1981) suggested that the remaining porin species increased so as to maintain a constant level of porin in the outer membrane, while Lugtenberg et.al., (1976) proposed that it is the overall level of outer membrane proteins which remains constant so that the loss of one protein allows all the other protein species to be incorporated to a higher level as space in the membrane becomes available. In view of the other physiological similarities between E. coli and S. typhimurium it seems likely that the porins of S. typhimurium are similarly regulated, so that analysis of the specificities of each porin species is difficult without a full range of deficient mutants. However, even without a full range of mutants it is clear that there are differences in specificities between the porin types. The loss of OmpD produces a greater reduction in Gly-Phe transport than does the loss of OmpC. The further loss of OmpC in NS4 from NS2

already deficient in OmpD might have been expected to produce a further decrease in Gly-Phe transport, however the loss of OmpC probably leads to increased production of OmpF which produces an increase in Gly-Phe transport. The complications produced by compensation of porin production should be surmountable by direct measurement of porin levels in a full range of porin-deficient mutants allowing the determination of the specificities of the different porin types.

On the basis of the results from this initial screening, the four strains H1 (Wild Type), H2 (*ompC*), H3 (*ompC*,*ompA*) and H4 (*ompB*) were selected for a kinetic analysis of peptide uptake to determine the effects of loss of porins and OmpA in more detail.

4.5 MICHAELIS MENTEN KINETIC ANALYSIS

The peptides Ala-Ala and Ala-Ala-Ala, which were used in this kinetic analysis, were chosen for a number of reasons: they are similar except in size so that any differences between them are likely to be caused by the size difference; their fluorescamine derivatives, although not amongst the highest yielding peptide derivatives, have a high fluorescence yield so that they are easy to measure at low concentrations; alanine has a low fluorescence yield at the pH used in these experiments so that amino acid interference will be low (Nisbet, 1980;see later) and they are rapidly transported by E. coli so that changes in transport rates should be sensitively measured. Ala-Ala-Ala-Ala was also tried as a further member of the homologous series but, although in other strains of E. coli it is transported rapidly, its rate of transport with these strains was low, making it inconvenient to use in this study.

Traces from the continuous flow assay (see section 1.4) give plots of fluorescence against time. The fluorescence is proportional to peptide concentration, therefore the trace represents peptide concentration against time. If the slope and fluorescence yield on the trace are measured, the rate of change of peptide concentration in the medium (peptide uptake) at the concentration represented by the fluorescence

yield can be determined. A number of values of velocity against peptide concentration can be determined at different points along the curve and these data can then be used to see if the uptake conforms to Michaelis Menten kinetics (see section 4.3), and if so to determine the Michaelis constant (Km) and the maximal velocity (Vmax) of the peptide transport system.

Initial rates of reaction at different substrate concentrations are usually used in enzyme kinetic studies. In this system however at the time of these studies the initial rate could not be measured directly because longitudinal mixing in the filter and tubing produces a 2 minute lag between time 0 and the steady state trace of uptake rate. Peptide uptake is accompanied by exodus of the amino acid products of peptide cleavage (Payne and Bell, 1979); the exodus of amino acids typically being measurable 10-15 seconds after peptide uptake starts, this being the time taken to "saturate" the amino acid pool; therefore amino acid exodus is occurring before the 2 minute period after which data are analysed.

The initial rate of transport (during the first few seconds) may be different from the subsequent steady rate because for example, there is no amino acid exodus and there will be a greater peptide concentration gradient into the

cell initially. If a fluorescence versus time trace is extrapolated back to zero time the peptide concentration indicated is typically the same as that added so that the initial transport is unlikely to be significantly different to the later measured rate. In principle amino acid exodus from the cell can cause errors in the calculation of peptide concentration and thus uptake rates from the trace, because amino acids can also form fluorescent products with fluorescamine. To convert the fluorescence value from the trace to peptide concentration it is necessary to know the fluorescence yields of a known peptide standard and its constituent amino acids and also the level of amino acid. However, experimental conditions are established to minimise any amino acid interference, mainly through controlling the pH of the reaction (see section 1.4). Thus, at pH 6.4 the molar fluorescent yield of an amino acid is typically .5-3% of that of an equimolar peptide solution (Nisbet, 1980). Therefore, amino acid interference can be ignored as negligible in most instances, although when peptide uptake is nearing completion the fluorescence contributed by the amino acids could form a large fraction of the total reaching 100% when all the peptide has been taken up. The contribution to the total fluorescence yield from the amino acid(s) at any point on the trace can be determined if the fluorescence yield of a standard solution of the amino acid(s) comprising the peptide under study is measured and it is assumed that

essentially all the amino acid(s) produced by cleavage undergoes exodus. The actual fraction of amino acid produced by peptide cleavage that undergoes exodus from the cell depends in part on the affinity of the amino acid permeases on the inner surface and also on how rapidly it is metabolised, the more quickly it is metabolised the less will be effluxed. This "metabolic potential" varies from one amino acid to another but an indication of it may be deduced from summing the intracellular pool levels and amounts of amino acid undergoing exodus observed during peptide transport, the more rapidly that an amino acid is metabolised the lower will be the sum of levels in the intracellular pool and external medium. Alanyl peptides were used in this study and all the available evidence indicates that the "metabolic potential" of alanine is relatively low so that essentially all of the alanine produced by cleavage during the incubation will be effluxed (early studies showed that this was subsequently reabsorbed and utilised; Payne and Bell, 1979). Thus knowing the level of amino acid interference this can be subtracted from the overall yield to give a corrected value for the peptide concentration (see fig 4.1). By compensating for the effect of amino acid interference, rates measured at the same peptide concentration should be comparable regardless of the initial peptide concentration (i.e. regardless of the amino acid concentration in the medium). Equation 8 has been used as the basis for a subroutine of the

Figure 4.1 Calculation of an amino acid correction factor

Apparent peptide yield (Ya) = True peptide yield (Yt) + Amino acid yield (Yaa)

$Ya = Yt + Yaa$

Assuming that all peptide taken up is cleaved and all resulting amino acid residues exported:-

Yaa = (Initial peptide yield (Yi) - True peptide yield (Yt))n.a

where n=number of amino acid residues in peptide

$a = \frac{\text{specific yield of amino acid}}{\text{specific yield of peptide}}$

$Yaa = (Yi-Yt)n.a$

$Yaa = n.aYi - n.aYt$

$Ya = Yt + n.aYi - n.aYt$

$Ya = Yt(1 - n.a) + n.aYi$

$Yt = \frac{Ya - n.aYi}{1 - n.a}$ .....(8)

4.6 RESULTS OF THE MICHAELIS MENTEN KINETIC ANALYSIS

The processed uptake data for strains H1-H4 are shown in Table 4.3.

program graph (see 2.6) which when provided with the values for initial yield, baseline, and the correction factor n.a., corrects the data from an assay for amino acid exodus.

The slope of the trace may be affected by a "smearing effect" which comes from longitudinal mixing during passage through the mixing chambers and delay coil so that the concentration of peptide measured at the fluorimeter at any one time does not exactly represent the concentration which was in the reaction vessel at the corresponding time of sampling, but is also a function of the peptide solution sampled just before and just after it. This "smearing" of the data tends to make each data point an average concentration over a time which is dependent on the mixing processes involved (the greater the mixing the longer the time over which averaging occurs). In practice, of course, by running assays under constant conditions any such variation can be eliminated. However, this "averaging" of each data point may under certain circumstances affect the shape of the curve produced: if the rate of uptake is constant the "smear" makes no difference and the fluorescence yield on the trace is a direct representation of the peptide concentration in the reaction vessel, but if the rate of uptake is changing significantly (as in some of the assays used in the Michaelis Menten analysis) then the effect of the "smear" will be to reduce the curvature of the resulting

trace. In experiments designed to test the effect of this "smear" by pumping diluent at a constant rate, into a reaction vessel containing peptide at a known initial concentration and thus producing a changing rate of dilution, there was a close correspondence between the calculated peptide concentration in the reaction vessel at any time and that represented on the trace (J.W. Payne, personal communication). The "smear" effect has therefore been ignored in this study, although it may have greater significance in studies using slower pumping rates where there might be greater longitudinal mixing.

To determine the rate of peptide uptake the slope of the trace must be expressed in terms such as, nanomoles of peptide transported mg cell protein^{-1} minute^{-1}. This is achieved by dividing the slope by the yield of a known standard of 1nmol of peptide and by multiplying by $2.2/A_{660}$ of the cell suspension (a cell suspension of A_{660} 2.2 units is equivalent to a cell protein level of 1mgml^{-1}, see section 2.3).

This process is performed automatically by another subroutine of the program "Graph" (see 2.6), which produces repeated estimates of rate of uptake along the length of the trace and the peptide concentration at which each rate occurred i.e. values of V and S. The program prompts for the

values necessary for the calculation of rates and concentrations e.g. initial peptide concentration, A_{660} of cells and baseline. After allowing the starting point of the repeated estimations to be adjusted using the movable pointers so that the initial equilibration parts of the trace are not included, the program estimates the slope and the average fluorescence yield of the trace between the two pointers and calculates the uptake rate and peptide concentration (V and S). The program then moves the pointers along to the next part of the trace, calculates V and S and continues to repeat the process until it reaches the end of the trace. In this way the program produces values for V and S taken from 64 second (the distance that the pointers are apart) non-overlapping portions of the trace. The data points produced are stored in output files so that the data from several runs either with the same or different peptide concentrations can be amalgamated and run through the program "Micmen" (see 2.6) to give estimates of Vmax and Km.

An example of the method of data gathering and processing is shown in Appendix A.

4.6 Results of the Michaelis Menten kinetic analysis
The processed uptake data for strains H1-H4 are shown in Table 4.3

It can be seen from the results in Table 4.3 that the values for the Vmax of uptake in all four strains are similar for a particular peptide. This lack of difference between the values for Vmax is important as it shows that the Vmax of transport is independent of outer membrane permeability. Such a result was predicted from theoretical considerations of outer membrane permeability and transport kinetics (Nikaido, 1979). The Vmax of a transport system is essentially a function of the permease protein(s) in the cytoplasmic membrane so that changes in outer membrane structure should not affect it.

The values obtained for apparent Km cannot be directly compared in the same way as the values for Vmax because Km values are not independent of their respective Vmax values. For any comparison to be meaningful it must be made between the values for Km/Vmax for each strain. The values obtained for Km/Vmax are also shown in Table 4.3.

There is only a small difference between the values for Km/Vmax for uptake of either Ala-Ala or Ala-Ala-Ala in strains H1 (wild type) and H2 (ompC). This indicates that the loss of OmpC porin from H2 has only a small effect on peptide permeability across the outer membrane. The further loss of OmpF porin from H4 does however lead to a significant increase in the values for Km/Vmax for uptake of both Ala-Ala

Table 4.3 Michaelis Menten kinetics for the uptake of Ala-Ala and Ala-Ala-Ala in outer membrane protein deficient strains H1-H4

	Ala-Ala			Ala-Ala-Ala		
Strain No.	Km	Vmax	Km/Vmax	Km	Vmax	Km/Vmax
H1	78	404	.19	31	232	.13
H2	108	366	.30	55	247	.22
H3	178	321	.55	182	177	1.03
H4	210	401	.52	258	248	1.04

Values for Km given as uM

Values for Vmax given as nmol peptide transported mg cellular protein^{-1} min^{-1}

and Ala-Ala-Ala. The loss of both porins causes an increase in the apparent Km of peptide transport i.e. a greater external concentration of peptide is needed to allow the same rate of peptide transport to occur; therefore we may conclude that the permeability of the outer membrane towards peptides has been lowered. The fact that the loss of OmpC alone has only a small effect, whereas the further loss of OmpF significantly decreases outer membrane permeability indicates that the OmpC porin may not be as important in facilitating the diffusion of peptides as is the OmpF porin. However, when one porin species is lost, production of the other porin is often increased presumably in an attempt to compensate for the loss (Nikaido, 1979). This process of compensation is not well understood. It has been suggested that the porin level is increased so as to maintain a constant level of porin molecules or a constant overall protein level in the outer membrane (Heller and Wilson, 1981; Lugtenberg et al., 1976). The situation is further complicated by the control of porin expression by the growth medium composition, for example, expression of OmpC is favoured by high osmotic strength and expression of OmpF by growth in nutrient broth (Van Alphen and Lugtenberg, 1977). L-broth was chosen as the growth medium in this study because its composition does not favour production of either OmpC or OmpF so that approximately equal amounts of both porins should be produced. This assumption was confirmed when outer membrane

protein fractions were prepared and separated by SDS-polyacrylamide gel electrophoresis (see 2.9 and Plate 4.1).

A decrease in the outer membrane permeability increases the contribution of the initial diffusion stage towards overall transport kinetics and as larger molecules diffuse more slowly than smaller molecules the former would be expected to be affected more by such a change. Thus, the loss of both porins in strain H4 increased the Km/Vmax ratio relative to the wild type approximately 8 times for Ala-Ala-Ala uptake but only 3 times for Ala-Ala uptake.

The loss of OmpA in H3 also produced a significant increase in the values for Km/Vmax for both Ala-Ala and Ala-Ala-Ala relative to H2 which indicates that OmpA is also important in facilitating peptide diffusion across the outer membrane.

The conclusions which can be drawn from the kinetic analysis are restricted by the limited range of strains which was available at the beginning of the study. To overcome this problem a wider range of strains was obtained from H. Nikaido which by then had become available. Dr Nikaido kindly sent a further 6 strains (JF568-JF703, see Table 4.1) which have been kinetically studied in the same way as

strains H1-H4.

The same two peptides, Ala-Ala and Ala-Ala-Ala were used in this further study but Gln-Gln was also used to investigate the role of polarity in peptide diffusion across the outer membrane as Gln-Gln is a highly polar peptide while remaining electrically neutral.

The results for strains JF568-JF703 are shown in Table 4.4.

The values for Vmax for all the strains were similar for a particular peptide as was the case with strains H1-H4, confirming that the outer membrane has no effect on the peptide permease *per se*.

The effect of loss of either OmpC porin or OmpF porin is clearly shown by comparing the values of Km/Vmax for strains 568, 701 and 703 (wild type, *ompC* and *ompF* respectively). The loss of OmpC from strain 701 causes a fall in the Km/Vmax, i.e. an increase in outer membrane permeability, presumably because when OmpC is missing from the outer membrane the level of OmpF rises, (see section 4.7), and as OmpF is more effective in facilitating diffusion then the overall membrane permeability rises. This is the case particularly with Gln-Gln which appears to diffuse through

Table 4.4 Michaelis Menten kinetic parameters for uptake of Ala-Ala, Ala-Ala-Ala and Gln-Gln in outer membrane protein deficient strains JF568-JF703

	Ala-Ala			Ala-Ala-Ala			Gln-Gln		
Str.	Km	Vmax	Km/Vmax	Km	Vmax	Km/Vmax	Km	Vmax	Km/Vmax
568	52	150	.35	45	119	.38	118	194	.61
694	77	167	.46	67	122	.55	171	182	.94
699	205	145	1.41	150	120	1.25	236	160	1.48
700	123	168	.73	171	201	.93	115	133	.86
701	30	165	.17	27	121	.22	16	179	.09
703	211	176	1.17	96	103	1.13	214	144	1.49

Values for Km given in uM

Values for Vmax given in nmol peptide transported mg cellular protein^{-1} min^{-1}

the OmpF pore very rapidly. The converse happens in strain 703 which is ompF and there is a similar large rise in the Km/Vmax for uptake for all three peptides used, indicating that the OmpC porin is not as efficient at facilitating peptide diffusion as the OmpF porin, confirming the results of the earlier part of the study. Gln-Gln uses the OmpC pore only as efficiently as Ala-Ala and Ala-Ala-Ala, so that its extra ease of diffusion through OmpF appears to be an example of pore specificity rather than generally faster diffusion by Gln-Gln.

Two more strains deficient in OmpA have been used here, 699 and 700 which are ompA and ompC, ompA respectively. Both strains show increases in Km/Vmax for all three peptides compared with the wild type, although the increases with 699 are greater than those for 700. This may be explained by the ompC mutation in strain 700 which has increased the level of OmpF porin in the outer membrane and thus increased the membrane permeability. Interestingly, the increase from wild type to 700 is least for Gln-Gln, endorsing the specificity suggested by the results of strains 701 and 703. These increases in Km/Vmax concomitant with loss of OmpA stress again the importance of the presence of OmpA in the outer membrane for efficient diffusion of peptides across the outer membrane.

Strain 694 is deficient in OmpC, OmpF and NmpA and constitutively expresses PhoE a pore-forming protein, which is normally phosphate induced. As the only pore-forming protein in the outer membrane in this strain, it does not appear to act as efficiently as OmpF, but rather more efficiently than OmpC when the values for Km/Vmax are compared with those for 701 and 703 respectively. Under the growth conditions used in this study the PhoE pore is not as efficient at allowing peptide diffusion as the wild type complement of pores (the Km/Vmax of transport is higher in 694 than 568 for all three peptides tested). This may be due to inhibition of diffusion through the PhoE pore by the phosphate ions used in the incubation buffer as has been reported by Overbeeke and Lugtenberg (1982) and Nikaido et al., (1983) who found that 2.5-10mM sodium phosphate inhibited diffusion of B-lactams through the PhoE pore by 8-40%.

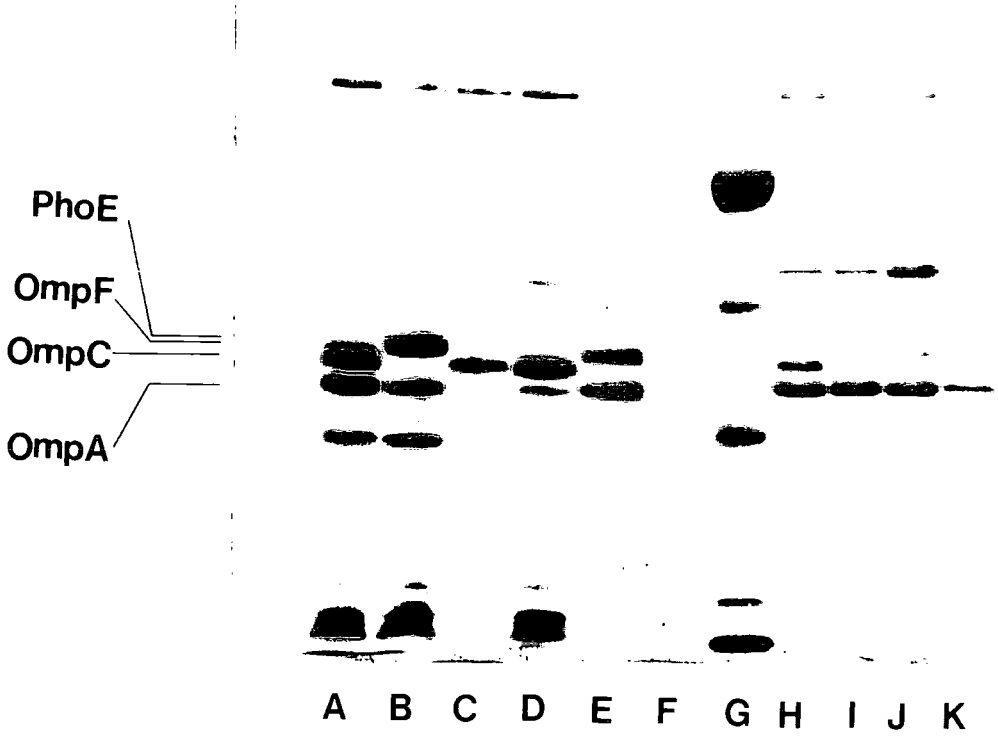
4.7 POLYACRYLAMIDE GEL ELECTROPHORESIS OF OUTER MEMBRANE PROTEINS

Outer membrane cell fractions were prepared and their constituent proteins separated by SDS-polyacrylamide gel electrophoresis as described in section 2.9. The outer membrane proteins from strains JF568-703 and H1-H4 are shown

in Plate 4.1. The two bands identified as OmpA represent the unmodified form of OmpA (apparent mol. wt. 27,000) and the heat modified form of OmpA (apparent mol. wt. 35,000). The samples were boiled for 5min immediately before loading onto the gel but there is still some OmpA present in its native form. PhoE appears to be expressed at a higher level than the porin proteins, if the relative intensities of the PhoE: OmpA and porin: OmpA bands are compared in the strains 568 and 694 (lanes A and B). PhoE may not therefore be as efficient at allowing diffusion as the kinetic analysis implies. Strains 699 and 700, which are both ompA (lanes C and D), show faint bands where the heat modified form of OmpA runs. There is either still low level expression of OmpA or a minor protein with the same electrophoretic mobility. Strains 701 and 703 (lanes E and F) show approximately equal levels of OmpA and porin (see section 4.6). This indicates that 701 and 703 express OmpF and OmpC respectively in approximately equal amounts (assuming that the production of OmpA does not change). Strain H1 (lane H) is a wild type strain and has a similar banding pattern to 568 (lane A) which is also a wild type. Strain H4 (lane I) is ompB and has lost both porins whereas strain H2 (lane J) still expresses OmpF.

Plate 4.1 Polyacrylamide gel elctrophoretic separation of the outer membrane proteins of porin- and OmpA-deficient strains of E. coli K12.

Lane A, JF568; B, JF694; C, JF699; D, JF700; E, JF701; F, JF703; G, protein standards; H, P400; I, P530; J, P400:6; K, P400:6hIr.


PhoE
OmpF
OmpC
OmpA
A B C D E F G H I J K

4.8 DISCUSSION

The results of the kinetic analysis of Ala-Ala and Ala-Ala-Ala uptake confirm and extend those derived from the initial screening of mutants. Nikaido (1979) warned that the measurement of transport rates at a single arbitrary substrate concentration would not allow any definite conclusions on the functions of porins to be drawn from the data produced. This present study shows that measurement of transport at a single, well chosen concentration near the apparent Km of the transport system being used can provide a rapid means of detecting mutations that have an effect on membrane permeability towards peptides. To investigate the precise effects of such a mutation requires a full kinetic analysis of uptake but the use of an effective rapid screening method such as that employed in this study enables many mutant strains to be tested for changes in outer membrane permeability. Only those mutants showing such a change need then be fully investigated. The results of the initial screen show that of all the mutant strains tested, only those defective in production of porin or OmpA had significantly reduced peptide transport. It is possible that some other outer membrane proteins do allow a low level of peptide diffusion to occur but that this would not have been detected in the initial screen, however any such proteins do not play a major role in peptide diffusion.

The kinetic analysis of peptide uptake indicates that the OmpF porin is more important in facilitating peptide diffusion than is the OmpC porin. Nikaido and Rosenberg (1983), using a liposome swelling assay have confirmed that peptides diffuse faster through OmpF than OmpC. From other studies there is some evidence that the OmpF porin is also a more efficient pore for such solutes as AMP, chloramphenicol, small sugars and B-lactams (Van Alphen et al. ,1978a; Lutkenhaus, 1977; Nikaido et al., 1983); furthermore in E. coli B/r, a strain which only produces one porin type, the porin produced is OmpF (Schmitges and Henning, 1976). From these observations one may infer that OmpF is the more physiologically important of the two porin species.

Nikaido and Rosenberg (1983) suggest that the difference in diffusion rates through the pores can be explained by the small difference in the diameters of the OmpC and OmpF pores, which they calculated as 0.54 and 0.58nm respectively from theoretical considerations of solute permeability (PhoE is calculated using the same method as 0.53nm). Benz and Hancock (1981) and Korteland et al., (1982) have calculated diameters of 1.4nm, 1.3nm and 1.2nm for OmpF, OmpC and PhoE respectively. If these small differences in pore size are confirmed then they must have some effect on the relative diffusion rate through the porins although whether this effect will be large enough to account on its own for the

differences in membrane permeability observed remains to be seen.

The PhoE protein has been shown in this study to produce a pore with broadly similar diffusion characteristics towards neutral peptides as OmpF and OmpC, confirming the results of Nikaido et al. (1983). The transport of negatively charged peptides e.g. Asp-Asp would be interesting to study as the PhoE pore has been reported to allow more rapid diffusion of anionic molecules than neutral molecules (Overbeeke and Lugtenberg, 1982) in contrast to the OmpC and OmpF pores (Nikaido et al., 1983).

The loss of OmpA from three strains has been shown here to reduce outer membrane permeability towards peptides. Manning et al., (1977) reported that amino acid uptake was also reduced by the loss of OmpA, although Nikaido (1979) suggested that this result may have been caused by low viability of OmpA deficient cells. When this specific point was checked, no such loss of viability was found in the strains used here (results not shown). Further evidence lies in the fact that the Vmax for peptide uptake was the same in ompA strains as in other strains. If a large proportion of ompA cells were dead, then the Vmax for uptake would be expected to be lower than in $OmpA^+$ cells. The increase in Km/Vmax does indeed seem to be correlated with a decrease in

membrane permeability. The OmpA protein extends right through the outer membrane as it acts as a receptor for phages at the outer surface (Van Alphen *et al.*, 1977; Datta *et al.*, 1977) and can be cross-linked to the peptidoglycan at the inner surface (Hall and Silhavy, 1979; Palva, 1979). It therefore is a potential candidate for a pore forming protein. There has been no study of the *E. coli* OmpA protein in liposomes to test for any pore forming activity, but the 33K protein from *Salmonella typhimurium* which is comparable to the OmpA protein has been tested and failed to show any activity (Nakae, 1976a). The OmpA protein has been shown to be strongly bound to the lipopolysaccharide *in vivo* (Van Alphen *et al.*, (1979) and may also be bound to the lipoprotein (Reithmeier and Bragg, 1977). These interactions may be essential in pore formation so that no activity would be detected in the liposome assay. It has also been suggested (Sonntag *et al.*,1978) that the OmpA protein is involved in maintaining tha outer membrane's structural integrity and as such may be important in maintaining other protein pores in an "open" configuration. Insertion of OmpA-lipoprotein lipopolysaccharide complexes into liposomes might help to show whether OmpA is capable of forming pores *per se* or is involved in stabilising pores formed by other proteins.

The specificities of the various pores in the outer membrane are still unclear. The evidence on this subject tends to be sporadic with different studies using a range of different solutes which are difficult to compare with each other. What is needed is a study using a standard procedure and a range of related substrates varying in size, charge etc. to provide a detailed picture of the specificities of each porin. The kinetic analysis of peptide uptake provides such a procedure and allows such a substrate range. The employment of the continuous flow fluorescamine assay (Nisbet and Payne, 1981) eliminates the need for radioactively labelled substrates which are not commercially available and difficult to synthesize and allows the use of the wide range of low cost, commercially available peptides.

A detailed knowledge of the specificities of each pore could be combined with our rapidly improving understanding of the control of porin production to provide answers to the fundamental questions of why the enterobacteriaceae need so many different pore forming proteins and how their complex regulation benefits the organism in its natural environment.

5 PEPTIDE TRANSPORT IN PSEUDOMONAS AERUGINOSA

5.1 INTRODUCTION

The Pseudomonads are a potentially interesting and important group to study. They occur in a great variety of environments and are able to use an unusually wide range of substrates for growth, so that they would be expected to possess the ability to efficiently utilise peptides. Pseudomonads also tend to be highly resistant to antibacterial agents, and novel ways of introducing toxic molecules into the cell using the "smugglins" concept (Matthews and Payne, 1975a) would be extremely useful. Little work has been done on peptide transport in Pseudomonads, there having been only two studies published to date by Cascieri and Mallete (1976a) and Miller and Becker (1978). Most of the work in both of these studies used growth of amino acid auxotrophs using peptides as amino acid sources to assay for peptide utilisation. As has already been discussed in 1.4, this type of assay, although useful as a preliminary test, is indirect and insensitive and the use of one of the direct assays for peptide transport, also discussed in 1.4, provides much more meaningful information. The only direct assay for peptide transport carried out so

far has been the measurement of uptake of Gly-Gly-[1-^{14}C]Ala in Pseudomonas putida by Cascieri and Mallete (1976a). The present study was undertaken to demonstrate peptide uptake in Pseudomonas aeruginosa by direct means and determine some of the characteristics of transport. The susceptibility of P. aeruginosa to a variety of peptide mimetic antibacterial agents was also tested.

5.2 STRAINS USED, GROWTH CONDITIONS AND METHODS

The strains used are shown in Table 2.1 and the growth conditions used are as described in section 2.2 and Table 2.2.

P. aeruginosa produces a mucoidal pellicle which tends to quickly block the Millipore filters used for cell harvesting. In order to harvest the large number of cells used for each run it was found to be necessary to use centrifugation (8,000g for 15min). Cells were spun down when growing exponentially, resuspended in the same volume of 50mM potassium phosphate buffer, pH7.2 (routinely 50ml) and spun again. The cells were then resuspended in a further 10ml of phosphate buffer and preincubated for 10min at 37°C before use.

Uptake of radioactively labelled peptides was measured as described in section 2.5. In the competition experiments, the rate determined in each assay was compared with the rate determined in a control assay using a further sample of the same cell suspension without competitor. The rate obtained in the assay with competitor is then expressed as a percentage of the rate without competitor. Competing peptides or amino acids were added immediately before the addition of radioactively labelled peptide. Gly-[U ^{14}C]Phe was supplied in aqueous solution with a specific activity of 12.8mCi mmol^{-1} and a concentration of 50uCi ml^{-1}. This solution was diluted with "cold" Gly-Phe to give a stock solution of 10mM Gly-Phe with a radioactive concentration of 5uCi ml^{-1}. Ala-Ala-[U ^{14}C]Ala was in solid form with a specific activity of 1.31uCi mg^{-1}. This was dissolved in water to give a stock solution of 10mM Ala-Ala-Ala with a radioactive concentration of 3.03 uCi ml^{-1}. Assays were routinely performed with an initial peptide concentration of 100uM (radioactive concentrations of 50nCi ml^{-1} for Gly-Phe and 30nCi ml^{-1} for Ala-Ala-Ala).

All other methods used were as described in Chapter 2.

5.3 EFFECT OF MEDIUM COMPOSITION ON GROWTH OF P. AERUGINOSA

Several different media have been used for the growth of Pseudomonas (Cascieri and Mallette, 1976a; Miller and Becker, 1978). In order to determine which of the media used best promotes the growth of P. aeruginosa , growth tests using various growth media were performed. Pseudomonas minimal medium (PMM), Pseudomonas Tris minimal medium (PTM) and the A+C medium of Davis and Mingioli were tested (for recipes see section 2.2). 10ml of each medium was inoculated with 0.1ml of an overnight culture of strain 6749 grown in A+C medium. The cultures were incubated at 37°C with rapid shaking and had their A_{660} measured periodically (see Figure 5.1) in a Bausch and Lomb Spectronic 20 spectrophotometer. A+C medium was found to be the best of the minimal media tested giving a doubling time of 55min. The growth of 6749 was also measured in L-broth when the doubling time was found to be 32min.

During the growth experiments samples of the cultures were periodically removed, serially diluted and plated out. A comparison of the cell number and its corresponding A_{660} gives a standard curve of cell number against A_{660} . A straight line relationship was found, with a cell suspension of A_{660} 1.0 units containing 1.9×10^9 cells which is a relationship similar to that found for E. coli (approx. 2×10^9 cells for an A_{660} of 1.0units; J.W. Payne, personal

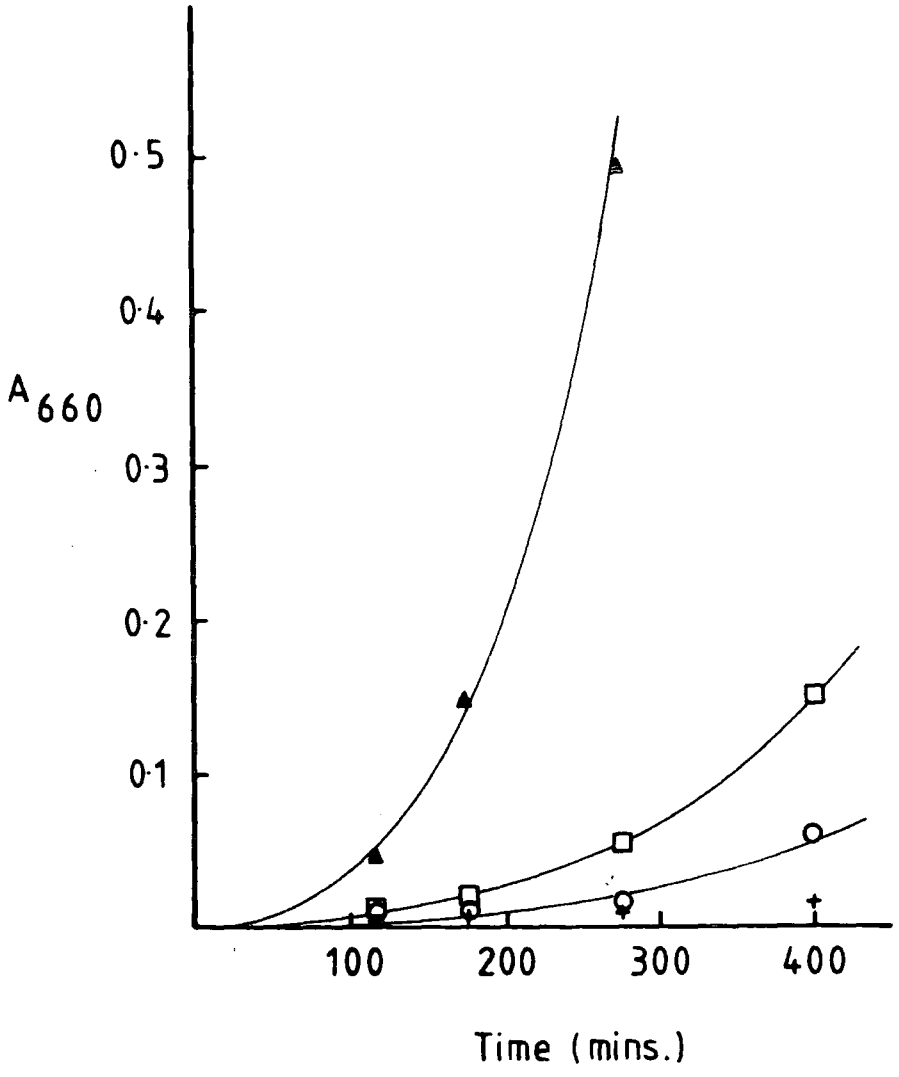


Figure 5.1 The effect of medium composition on growth of P. aeruginosa □, Davis and Mingioli; O, Pseudomonas minimal medium; +, Pseudomonas Tris medium; ▲, Luria broth; (see section 2.2 for composition of media).

communication). No difference in the relationship between cell number and A_{660} was found between cells grown in different media.

On the basis of these results, A+C and L-broth were selected as the minimal and complex medium respectively.

5.4 DIRECT MEASUREMENT OF PEPTIDE UPTAKE IN P. AERUGINOSA USING THE DANSYL CHLORIDE ASSAY

Introduction

Of the fluorescence assays in use (see section 1.4), the dansyl chloride assay provides the most information and can be used to simultaneously monitor uptake and exodus of both amino acids and peptides. It was decided to begin the investigation with dansyl chloride assays of uptake.

Methods

The dansyl chloride assays were performed as described in section 2.5, except that harvesting was by centrifugation instead of filtration.

Results

Initial experiments to detect the uptake of Gly-Sar, Ala-Ala and Ala-Ala-Ala used cell suspensions of strain 6749 grown in minimal medium of A_{660} 0.5-1.0. Samples were taken over a 15min period but there was no detectable uptake of any of the peptides, either from the medium or into cell extracts. This lack of activity could be either due to a low constitutive rate of transport or a repressed inducible transport system. In an attempt to produce a more active transport system, cells were grown in L-broth or minimal medium plus 1% tryptone. These cell suspensions were used with an A_{660} of 1.0-1.2 and samples were taken over 60min. The same three peptides were tested again and there was still no detectable uptake from the medium filtrates but traces of Gly-Sar were seen in the cell extracts. Importantly, however this dansyl technique showed there was no evidence for any peptide cleavage in the external medium, indicating that P. aeruginosa does not produce extracellular or periplasmic peptidases (at least against those peptides tested. The traces of Gly-Sar appearing inside the cells showed that uptake was occurring but that the dansyl chloride assay was not sensitive enough to measure disappearance from the medium. It was therefore decided to use the more sensitive manual fluorescamine assay in further experiments.

Discussion

Initial experiments using dansyl chloride failed to show any uptake activity when assaying for removal of peptide from the medium. If a peptide were to be taken up at 0.5 nmol min^{-1} mg cellular $protein^{-1}$ (using the results of the later fluorescamine experiments), and the cell suspension contained 0.5mg cellular protein ml^{-1} (equivalent to an A_{660} of 1.1), then the peptide uptake expected would be 0.25nmol ml cell $suspension^{-1}$ min^{-1}. Over one hour, 15nmol of peptide should disappear from each ml of cell suspension leaving 85nmol still present (100uM initial concentration=100nmol ml^{-1}). This would be at the limit of resolution of the dansyl chloride assay using only simple visual inspection of dansyl plates.

The lack of detectable Ala-Ala and Ala-Ala-Ala in cell extracts can also be accounted for. The intracellular peptidase activity in Pseudomonas has been shown to be up to 1000 times higher than the peptide transport activity shown here (Cascieri and Mallete, 1976b; Haas, et al., 1981) so that any peptide transported into the cell would be rapidly hydrolysed to its amino acid constituents.

The detection of Gly-Sar in the cell extracts does however provide the first demonstration of intact peptide uptake in

Pseudomonas. Cascieri and Mallette (1976b) failed to detect any peptidase activity towards Gly-Sar, although the assay used would have failed to detect any activity below 2nmol amino acid formed min^{-1} mg cellular $protein^{-1}$. Only traces of Gly-Sar were seen here in cell extracts after incubation for one hour. If, as discussed above, one ml of cell suspension takes up 15nmol of Gly-Sar from the medium in one hour, then each sample of the 1h incubation cell extracts used in the dansylation reaction (100ul) would contain 1.5nmol of Gly-Sar. Only half of this is spotted onto each polyamide sheet, therefore 0.75nmol of Gly-Sar should have been present on these chromatograms. The minimum level of detection of the dansyl chloride assay is approximately 0.2nmol of peptide (J.W. Payne, personal communication). It is difficult to quantify such low levels of peptide from a visual inspection of the plates, so that the levels of Gly-Sar observed are consistent with those calculated from the results of the fluorescamine assays carried out later.

Although the dansyl chloride assays did not provide as much information about uptake as had been hoped, they demonstrated that no extracellular or periplasmic peptidases active towards the peptides tested are present in P. aeruginosa, as no cleavage products of the peptides used in each assay were detected even after one hour of incubation. This lack of external peptidase activity allows the use of

the more sensitive fluorescamine assay.

5.5 EFFECT OF NITROGEN SOURCE ON PEPTIDE UPTAKE IN P. AERUGINOSA

Introduction

The ability to utilise every available nutrient source efficiently would be consistent with the known wide range of habitats which P. aeruginosa occupies. One might expect, therefore, that peptide transport would occur at a rate comparable to that in E. coli when P. aeruginosa was growing in an environment containing peptides. Such a transport system might be inducible only under certain conditions which had not been provided in the studies undertaken so far. If a higher rate of peptide transport were possible by varying the growth conditions, it would greatly facilitate the rest of this study. Therefore before any further work was undertaken, on the assumption that nitrogen regulation af peptide transport might occur, cells were grown in media containing several different nitrogen sources to assess their influence on the rate of peptide transport. The rate of peptide uptake was measured using the manual fluorescamine assay and the growth rate of cells in each medium was monitored.

Methods

Overnight cultures grown in A+C medium were used to provide inocula for experimental cultures in media containing various nitrogen sources. These cultures were harvested during exponential phase and used in the manual fluorescamine assay as previously described (see section 5.2).

Growth rates were monitored by measuring the A_{660} of cultures in a Bausch and Lomb Spectronic 20 spectrophotometer.

Results

The uptake of Gly-Phe or Ala-Ala-Ala was measured after growth in a medium containing proline, Pro-Gly, peptone or ammonium sulphate as the nitrogen source. For the results see Table 5.1. The results of the growth measurements are shown in Figure 5.2.

Discussion

Growth in a medium containing either ammonium sulphate and/or proline as sole nitrogen source did not stimulate peptide uptake over that on ammonium sulphate alone. Growth with proline and Pro-Gly did not cause any marked increase in

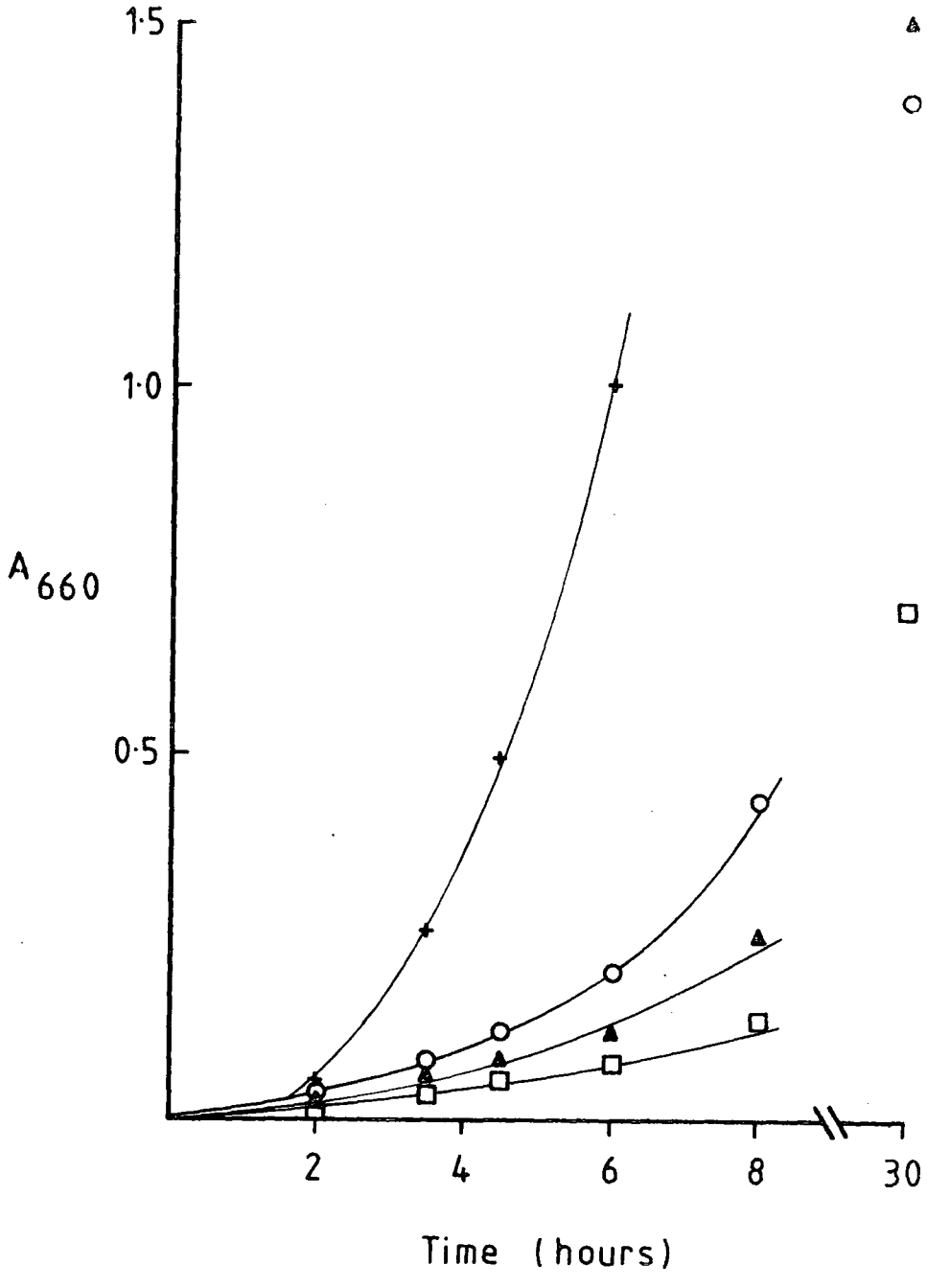


Figure 5.2 Effect of nitrogen source on growth of P. aeruginosa. + , 1% (w/v) tryptone; O , ammonium sulphate 1mg/ml; Δ , proline 5mg/ml; □ , Pro-Gly 1mg/ml. Basal growth medium was A+C without addition of ammonium sulphate.

Table 5.1. Influence of nitrogen source on peptide uptake in P. aeruginosa

Growth medium composition	Peptide uptake	
	Gly-Phe	Ala-Ala-Ala
+ NH_4	0.5	0.4
+ Pro	0.5	0.5
+ NH_4, Pro		0.4
+ Pro, Pro-Gly	0.7	
+ Peptone	0.5	

The basal growth medium was A+C without addition of ammonium sulphate.

Supplements were added as follows:

ammonium sulphate, 1mg ml^{-1} ; proline, 5mg ml^{-1} ; Pro-Gly, 1mg ml^{-1} ; peptone, 1% (w/v).

Uptake rates are expressed in nmol min^{-1} mg cellular $protein^{-1}$.

All values are the mean of 2-3 experiments and have a S.E. of approximately 0.15nmol min^{-1} mg^{-1} .

peptide transport either. The use of a peptide as the sole nitrogen source was precluded by the large amount of peptide required to grow 50ml of cells (250mg per assay). Peptone, which contains large amounts of small peptides, was also used, but this too failed to stimulate peptide transport (this confirms the results of the dansyl chloride assays when peptone was used in the growth medium and peptide transport was very slow or undetectable). Although it would be desirable to extend these studies, perhaps using peptide with amino acid auxotrophs, from these results it would appear that the rate of peptide transport observed is constitutive and perhaps the maximum of which this organism is capable.

The growth measurements indicate that in P. aeruginosa, proline and Pro-Gly are much poorer nitrogen sources than ammonium sulphate, despite there being three times as much nitrogen present in the media in amino acid form than in ammonium form. The effect of peptone on growth is more difficult to interpret because of the various nitrogen containing compounds which are present. Using the analysis presented in the Difco manual (1953) a 1% peptone solution should contain approximately 0.9mg ml^{-1} nitrogen in amino acids and 0.4mg ml^{-1} nitrogen in dipeptide form, however there may also be up to 0.3mg ml^{-1} of nitrogen in ammonium form so that this is likely to be the major source of utilisable nitrogen in the medium.

5.6 DIRECT MEASUREMENT OF PEPTIDE UPTAKE IN P. AERUGINOSA USING THE MANUAL FLUORESCAMINE ASSAY

Introduction

The manual fluorescamine assay is more sensitive than the dansyl chloride assay and it was a logical progression to start using it when the dansyl chloride assay proved too insensitive to detect peptide transport in *P. aeruginosa*. The dansyl chloride assay had, however, indicated the absence of any extracellular or periplasmic peptidase activity. This is important, as the fluorescamine assay fails to distinguish between peptide transport *per. se.* and superficial peptidase activity (see section 1.4).

Methods

Cells were grown in L-broth and harvested as described in section 5.2. Manual fluorescamine assays were performed as described in section 2.5.

Results

Samples were taken from cell suspensions shaken in a water bath at 37°C, of A_{660} 1.0-1.5 over a period of 90min (initial peptide concentration 100uM) and uptake was demonstrated for

a number of peptides (see Table 5.2).

Discussion

Use of the manual fluorescamine assay was successful in demonstrating peptide uptake in P. aeruginosa. Transport of di- and oligopeptides was shown up to tetraalanine. No decrease in the rate of uptake was observed when going from a tri- to a tetrapeptide. This result endorses the conclusion of Miller and Becker (1978) (also in P. aeruginosa) but contradicts that of Cascieri and Mallette (1976a) who found that a methionine containing tetrapeptide supported the growth of a methionine auxotroph of P. putida at a rate 3 times slower than that suppported by a methionine containing tripeptide, despite similar peptidase activities towards tri- and tetrapeptides. This could be a reflection of a much lower rate of tetrapeptide transport in P. putida, as Cascieri and Mallette concluded, however, when peptide transport is only just sufficient to provide enough amino acid source to support growth then a small decrease in transport rate can cause a large decrease in growth rate, and this may be the case here.

The uptake of Gly-Sar which was shown by the dansyl chloride assay is confirmed by these results. No uptake was detected for Gly-DVal, suggesting that peptide transport in P

Table 5.2. Peptide uptake by P. aeruginosa measured by using the manual fluorescamine assay

Peptide	Uptake
Ala-Ala	0.6
Ala-Ala-Ala	0.4
Ala-Ala-Ala-Ala	0.5
Leu-Leu-Leu	0.6
Gly-Gly-Pro	0.8
Gly-Sar	0.6
Gly-Phe	0.5
Gly-Val	0.7
Gly-DVal	ND

Uptake rates given as nmol peptide min^{-1} mg cellular $protein^{-1}$.

All values are the mean of 2-3 experiments and have a S.E. of approximately 0.15nmol min^{-1} mg^{-1}

All initial peptide concentrations were 100uM

aeruginosa has some stereospecificity towards L- amino acid residues, as has been shown to occur in the other bacterial species studied (Payne, 1980). The stereospecificity of peptide transport in P. aeruginosa is dealt with in more detail in section 5.11.

In order to check that strain 6749 is not atypically slow at transporting peptides (it might possibly be peptide transport deficient already), strains P2 and P3 (see Table 2.1) were also tested for uptake of Ala-Ala and Ala-Ala-Ala and gave similar rates of uptake to those of strain 6749 (data not shown).

These results show that P. aeruginosa does transport a range of peptides, but that the rates observed are low compared with many other previously studied bacteria.

5.7 DIRECT MEASUREMENT OF PEPTIDE UPTAKE IN P. AERUGINOSA USING RADIOACTIVELY LABELLED PEPTIDES

Introduction

The rates of transport measured in this study are near the minimum rates reliably detectable by the fluorescamine assay. Measurements of the effects of metabolic inhibitors on uptake and calculation of kinetic parameters is therefore difficult using this method. In addition information on the number and nature of peptide permeases in P. aeruginosa would also be difficult to obtain using the fluorescamine assay as competition experiments cannot be performed. In view of these problems it was decided to use radioactively labelled peptides to study these aspects of peptide transport. The only report to date of the use of this assay in Pseudomonas has been that of Cascieri and Mallette (1976a) who measured the uptake of Gly-Gly-[1-^{14}C]Ala in P. putida. The uptake of glycine and phenylalanine was also measured to provide a comparison between the rates of uptake for amino acids and peptides and a comparison between the rates obtained in this study and those obtained in previous studies of amino acid transport in P. aeruginosa (Kay and Gronlund, 1969).

Methods

Table 5.3. Peptide uptake in P. aeruginosa measured using both the radioactively labelled peptide assay and the manual fluorescamine assay

Peptide	Rate	Readings	S.E.	S.E./Rate
Ala-Ala-Ala (R)	0.57	6	0.07	0.12
Ala-Ala-Ala (F)	0.4	3	0.10	0.25
Gly-Phe (R)	0.34	6	0.05	0.14
Gly-Phe (F)	0.5	6	0.12	0.24
Gly (R)	0.2	2		
Phe (R)	0.9	2		

Rates given as nmol peptide (amino acid) min^{-1} mg cellular $protein^{-1}$

(R)= Radioactively labelled peptide assay

(F)= Manual fluorescamine assay

S.E.= Standard error

Uptake of radioactively labelled peptide or amino acid was measured as described in section 2.5, except for cell harvesting which is described in section 5.2.

Results

Aliquots of cell suspension were removed at the beginning and end of each experiment to detect any changes in the total number of counts present in the incubation medium (as described in section 2.5). No change in the total number of counts was observed, indicating that under the conditions used in the assay, P. aeruginosa does not cause any loss of counts to the air through decarboxylation of [U-^{14}C]Phe or [U-^{14}C]Ala. Such a loss of counts has been shown to affect the rates obtained from uptake assays using some radioactively labelled peptides in E. coli and other microorganisms (Payne and Nisbet, 1980a; Nisbet, 1980).

The rate of uptake of Ala-Ala-[U-^{14}C]Ala and Gly-[U-^{14}C]Phe at an initial concentration of 100uM was measured six times for each peptide. The means of these results together with their standard errors are shown in Table 5.3. Also included for comparison are the values for uptake of Ala-Ala-Ala and Gly-Phe obtained from the manual fluorescamine assay. Uptake rates for radioactively labelled glycine and phenylalanine are also shown. The rates of

uptake of radioactively labelled Ala-Ala-Ala and Gly-Phe were also measured at initial concentrations of 20 and 10uM. These results were plotted using the Lineweaver-Burke transformation of the Michaelis-Menten equation (see Figure 5.3) and the values for Km and Vmax calculated from the y-axis and x-axis intercepts respectively. The values obtained are shown in Table 5.4.

Discussion

If each standard error for peptide uptake is expressed as a percentage of the mean uptake rate for that peptide, it can be seen that the standard error obtained when using the manual fluorescamine assay is approximately twice as large as that obtained from the radioactively labelled peptide transport assay (25% compared to 13%). This confirms that using radioactively labelled peptides is a more sensitive and precise way of monitoring peptide uptake under these conditions.

Any amino acid exodus lowers the apparent rate of peptide uptake measured using the radioactively labelled peptide assay while not affecting the fluorescamine assay to the same extent because the radioactively labelled peptide assay does not discriminate between amino acids and peptides while the fluorescamine assay does (see section 1.4). The similarity

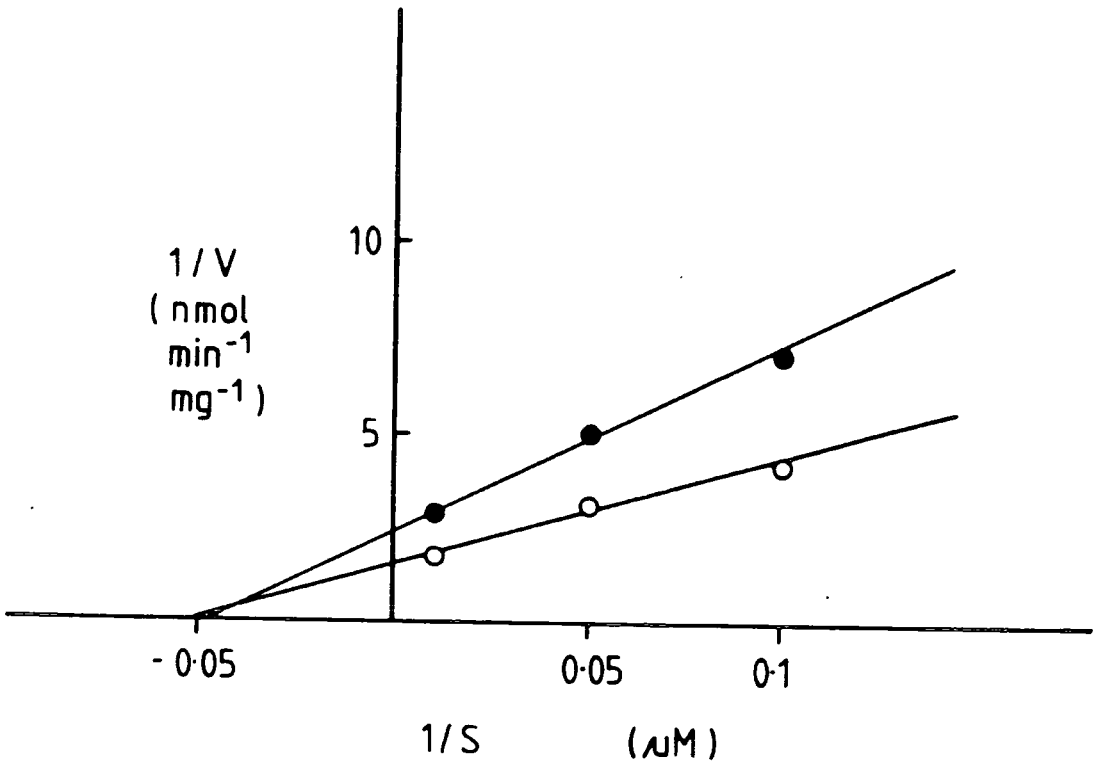


Figure 5.3 Double reciprocal plot of the uptake of Gly-Phe (●) and Ala-Ala-Ala. (○)

Table 5.4. Kinetic parameters for peptide uptake in P. aeruginosa

Peptide	Vmax	Km
Ala-Ala-Ala	0.65	18
Gly-Phe	0.42	19

Values for Vmax given in nmol peptide min^{-1} mg cellular $protein^{-1}$

Values for Km given in uM

between the rates obtained by both methods indicates that little if any amino acid exodus occurs during peptide uptake in P. aeruginosa.

The values for the Km of peptide uptake calculated here are similar to, or a little higher than those obtained for peptide transport in E. coli by other workers using the same methods (Cowell, 1974; Staros and Knowles, 1978). Yoshimura and Nikaido (1982) found that the values for the growth Km (the substrate concentration at which the growth rate is half maximal) of a number of carbon sources were higher in P. aeruginosa than in E. coli although the data were difficult to obtain because of a growth lag and cell autolysis during culture dilution. Yoshimura and Nikaido (1982) concluded that low permeability of the outer membrane was responsible for the high growth Km values which they observed. The effects of EDTA and polymyxinB, which are outer membrane disruptive agents in P. aeruginosa (Brown, 1975), are studied in section 5.12 to further investigate the role of the outer membrane in transport.

The values for uptake of phenylalanine and glycine are similar to those obtained for peptide transport, indicating that peptides are not especially poorly transported in P. aeruginosa. The rates are also similar to those obtained by Kay and Gronlund (1969) who reported that the rates of uptake

of 18 amino acids in P. aeruginosa varied between 0.2 and 8nmol min^{-1} mg cellular $protein^{-1}$.

5.8 AMINO ACID AND PEPTIDE TRANSPORT ARE SEPARATE IN P. AERUGINOSA

Introduction

In order to demonstrate peptide transport *per se* and not merely extracellular cleavage followed by amino acid uptake, it is necessary to show that peptides and their constituent amino acids do not compete for uptake (see section 1.3). Evidence from Miller and Becker (1978) and the dansyl chloride assays performed in this study (section 5.4) indicates that there is no extracellular or periplasmic peptidase activity in *P. aeruginosa* either, but as yet there has been no direct demonstration of the separation of amino acid and peptide transport.

Methods

The competition assays were performed as described in section 5.2.

Results

Concentrations of 100uM and 1mM of alanine and phenylalanine were used as competitors for uptake with Ala-Ala-Ala and Gly-Phe respectively (see Table 5.5). No

Table 5.5. Competition for uptake between amino acids and peptides in P. aeruginosa

Peptide	Competitor	% uptake remaining
Ala-Ala-Ala		100
Ala-Ala-Ala	Ala (100uM)	102
Ala-Ala-Ala	Ala (1mM)	97
Gly-Phe		100
Gly-Phe	Phe (100uM)	100
Gly-Phe	Phe (1mM)	93

All peptide concentrations 100uM

competition for uptake between peptides and amino acids was observed.

Discussion

These results confirm earlier observations (Miller and Becker, 1978; Haas et al., 1981; this study, section 5.4) that there is no extracellular peptidase activity in P. aeruginosa and indicate that intact peptides are recognised by the permease and only hydrolysed later. The uptake of intact Gly-Sar shown in section 5.4 also indicates that peptide hydrolysis is not an obligatory step in peptide transport in P. aeruginosa as has been suggested by Miller and Becker (1978), but occurs as a separate reaction after the intact peptide has been released from the permease.

5.9 COMPETITION BETWEEN DI- AND OLIGOPEPTIDES FOR UPTAKE IN P. AERUGINOSA

Introduction

No study of competition between di- and oligopeptides has been reported for P. aeruginosa. The only study carried out on a Pseudomonad was that of Cascieri and Mallette (1976a) who showed that tripeptides did not inhibit growth of a methionine auxotroph of P. putida on Gly-Met but that other dipeptides could. They concluded that there were separate systems for di- and oligopeptides in P. putida.

Methods

The competition assays were performed as described in section 5.2.

Results and discussion

Competition for uptake between Ala-Ala-Ala and Gly-Phe and a range of di- and oligopeptides was measured (see Table 5.6). All the oligopeptides tested competed for uptake with Ala-Ala-Ala indicating that they share a common transport system. The largest peptide shown to compete with Ala-Ala-Ala was hexaalanine. Miller and Becker (1978) showed

Table 5.6. Competition for uptake between di- and oligopeptides in P. aeruginosa

Peptide	Competitor	Conc. of competitor	% uptake remaining
Ala-Ala-Ala			100
Ala-Ala-Ala	Leu-Leu-Leu	100uM	91
Ala-Ala-Ala	Leu-Leu-Leu	1mM	48
Ala-Ala-Ala	Hexaalanine	100uM	81
Ala-Ala-Ala#	Hexaalanine	200uM	60
Ala-Ala-Ala	Tetraalanine	100uM	84
Ala-Ala-Ala	Gly-Phe	100uM	95
Ala-Ala-Ala	Gly-Phe	1mM	72
Ala-Ala-Ala	Ala-Ala	100uM	100
Ala-Ala-Ala	Ala-Ala	1mM	42
Ala-Ala-Ala	Leu-Leu	1mM	60
Gly-Phe			100
Gly-Phe	Ala-Ala	100uM	43
Gly-Phe	Leu-Leu	1mM	29
Gly-Phe	Ala-Ala-Ala	100uM	93
Gly-Phe	Ala-Ala-Ala	1mM	80
Gly-Phe	Leu-Leu-Leu	1mM	50
Gly-Phe	Val-Val-Val	1mM	38

Ala-Ala-Ala and Gly-Phe used at 100uM unless stated.

Ala-Ala-Ala used at 20uM, uptake calculated relative to 20uM Ala-Ala-Ala without competitor.

that pentamethionine could support the growth of P. aeruginosa met and it is now clear that peptides as least as long as hexapeptides can be transported.

All the dipeptides tested also competed with Ala-Ala-Ala for uptake. Most peptide permeases that can transport oligopeptides can also transport dipeptides (Payne, 1980) presumably because such permeases have little specificity for the C-terminus which can vary in position depending on the chain length of the peptide. Both dipeptides tested competed with Gly-Phe for uptake indicating that they too share a common transport system. Interestingly, however, all the oligopeptides tested also competed with Gly-Phe for uptake. The results presented here seem, at first, to suggest that there is only one peptide permease in P. aeruginosa which handles both di- and oligopeptides. Equally consistent with these results would be two (or more) peptide transport permeases with overlapping specificities. This seems to be the case in E. coli, where the dipeptide permease and oligopeptide permease both transport di- and oligopeptides (see section 3.6). There are several ways of determining how many transport systems are present:-

1) By isolating mutants deficient in peptide transport and measuring the residual uptake activity (if any). Attempts to isolate peptide deficient mutants are described in section 5.10.

2) Transport kinetic data can be transformed so that it is possible to distinguish uptake by one or more systems (e.g. the Inui-Christensen plot, Inui and Christensen, 1966). These methods however require data which are more reproducible than it is possible to obtain in this instance.
3) Reciprocal competition studies can be undertaken in which the competition of substrate A with substrate B and then substrate B with substrate A is measured. If only one transport system is present then one substrate should show a consistently higher affinity than the other one regardless of which is the competitor. If there is more than one transport system then this need not be the case, for instance, if A has a high affinity for transport system 1 and B has a low affinity for it, then B will compete poorly with A. If B has a high affinity for transport system 2 and A a low affinity for it, then A will also compete poorly with B. Therefore the apparent affinity for transport depends on which peptide is the competitor. This seems to be the case for Gly-Phe and Ala-Ala-Ala. At a 10:1 molar ratio, Gly-Phe only inhibits Ala-Ala-Ala uptake by 28% , Ala-Ala-Ala, also at a 10:1 molar ratio, only inhibits Gly-Phe uptake by 20% instead of the 95-98% expected. This indicates that there is more than one peptide uptake system in P. aeruginosa with each capable of transporting both di- and oligopeptides.

5.10 RESISTANCE OF P. AERUGINOSA TO PEPTIDE MIMETIC ANTIBIOTICS

Introduction

Peptide mimetic antibiotics have been shown to be toxic to a range of microorganisms (Ringrose, 1980) and several have been used successfully in this study (see section 3.3) to isolate peptide permease deficient mutants in E. coli. The isolation of such mutants in P. aeruginosa would greatly facilitate the study of the number and specificities of the peptide permease(s) present. Pseudomonas species possess a high level of intrinsic resistance to many antibiotics; if peptide mimetic antibiotics were to be toxic towards Pseudomonads, this would represent a potentially important advance in Pseudomonad chemotherapy.

Methods

The lawn inhibition zone assay was used (see section 2.4) with approximately $2x10^8$ cells spread per plate.

Results

See Table 5.7 for the peptide mimetic antibiotics used. Initial experiments used 10ul of peptide solutions of

Table 5.7. Peptide mimetic antibiotics used against P. aeruginosa

Ala-aminoxypropionic acid

Ala-Ala-aminoxypropionic acid

Pro-Ala-aminoxypropionic acid

Ala-aminoethylphosphonic acid

Ala-Ala-aminoethylphosphonic acid

Orn-Orn-Orn

Norleu-Norval

concentration 1-20mM. None of these proved inhibitory to growth. Later experiments used 1-2mg of solid peptide loaded directly onto the centre of the plate, but these too were not inhibitory to growth.

Discussion

It is clear from these results that *P. aeruginosa* is highly resistant to peptide antibiotics known to inhibit other bacteria *e.g.* enterobacteria. The range of antibiotics used makes it unlikely that the cells are resistant to each toxic moiety *per se*, it is more likely that transport of these peptides occurs so slowly that the level of antibiotic accumulated inside the cells is insufficient to cause significant inhibition. This lack of inhibition precluded the isolation of transport deficient mutants by this means here.

5.11 STEREOSPECIFICITY OF PEPTIDE TRANSPORT IN P. AERUGINOSA

Introduction

There have been no reports concerning the stereospecificity of peptide transport in P. aeruginosa. In growth studies using a methionine auxotroph of P. putida Cascieri and Mallete (1976a) showed that a D-amino acid residue in either position in a dipeptide or the N-terminal position in a tripeptide prevented competition with a Met containing di- or tripeptide respectively. The effects of D-amino acid residues in other positions in tripeptides were not studied. Here, various stereoisomers of Ala-Ala-Ala and Ala-Ala have been used in competition studies with LAla-LAla-LAla and Gly-LPhe respectively.

Methods

The competition studies were carried out as described in section 5.2.

Results and discussion

The results of the competition studies for uptake between the peptide stereoisomers are shown in Table 5.8. Neither DAla-DAla-LAla nor DAla-DAla-DAla competed for uptake with

Table 5.8. Competition for uptake between peptide stereoisomers in P. aeruginosa

Peptide	Competitor	% uptake remaining
Ala-Ala-Ala		100
Ala-Ala-Ala	Ala-DAla-DAla	70
Ala-Ala-Ala	DAla-DAla-Ala	100
Ala-Ala-Ala	DAla-DAla-DAla	100
Gly-Phe		100
Gly-Phe	Ala-Ala (100uM)	43
Gly-Phe	Ala-DAla	94
Gly-Phe	DAla-Ala	100
Gly-Phe	DAla-DAla	100

All peptide concentrations 100uM

All competitor concentrations 1mM unless stated

All L-residues unless stated

Ala-Ala-Ala whereas LAla-DAla-DAla did compete although not very strongly. This indicates that the requirement of the peptide permease for an N-terminal L-amino acid residue is absolute whereas in other positions D-amino acid residues can be tolerated, but that they reduce the binding affinity between the peptide and the permease. In E. coli there is a requirement for the N-terminal and second residues of a tripeptide to be in the L-configuration (Payne,1980).

Only LAla-LAla was found to compete with Gly-Phe for uptake. This shows that dipeptide transport in P. aeruginosa is specific for L-amino acids in both residues as is dipeptide transport in E. coli (Payne, 1980). These results provide further evidence for there being two separate peptide transport systems in P. aeruginosa, one stereospecific for the first and second residues of a peptide and with a high affinity for Gly-Phe and one with a less strict requirement for the second residue of a peptide and a high affinity for Ala-Ala-Ala.

5.12 THE EFFECT OF METABOLIC INHIBITORS ON PEPTIDE TRANSPORT IN P. AERUGINOSA

Introduction

Peptide transport has been shown to be coupled to phosphate bond energy in E. coli (Cowell, 1974; Payne and Bell, 1979) and to a proton gradient in Saccharomyces cerevisiae and Streptococcus faecalis (Nisbet, 1980). The two types of energisation of transport can be distinguished by the use of metabolic inhibitors. Substances such as carbonylcyanide m-chlorophenylhydrazone (CCCP) act as protonophores destroying the transmembrane proton gradient (CCCP) or alternatively, substances such as N-N′ dicyclohexylcarbodiimide (DCCD) act as inhibitors of the ATPase used to produce the gradient and thus both effectively inhibit transport systems linked to proton gradients. Other inhibitors such as arsenate, which is a phosphate antagonist, interfere with glycolytic and respiratory ATP production and thus inhibit phosphate bond linked transport. The inhibition of transport by one type of inhibitor but not by the other is strong evidence for transport being linked to one or other form of energisation.

Cascieri and Mallette (1976a) reported that uptake of radioactively labelled Gly-Gly-[1-^{14}C]Ala in P. putida was

abolished by addition of 5mM 2,4-dinitrophenol (DNP). This result implies that peptide transport in P. putida may be energised by a proton gradient as DNP is a protonophore and destroys the transmembrane proton gradient.

The effects of both arsenate and DCCD on the uptake of Gly-Phe and Ala-Ala-Ala in P. aeruginosa are described here.

Methods

Cells were harvested and resuspended as described in section 5.2 except that cells to be used with arsenate were resuspended in TrisHCl buffer (50mM, pH7.2) instead of phosphate buffer (as phosphate and arsenate are antagonists, the high phosphate concentration in the buffer would decrease any inhibitory effect of arsenate). Uptake rates of cells with added arsenate were compared with rates obtained from cells without arsenate also incubated in Tris buffer. Inhibitors were added to cell supensions 10min before the addition of radioactively labelled peptide.

Results

The effects of arsenate and DCCD on Ala-Ala-Ala and Gly-Phe uptake are shown in Table 5.9. DCCD has no inhibitory effect on either Ala-Ala-Ala uptake or Gly-Phe

Table 5.9. Effects of arsenate and DCCD on peptide uptake in P. aeruginosa

Peptide	Inhibitor	% uptake remaining
Ala-Ala-Ala		100
Ala-Ala-Ala	DCCD (1mM)	103
Ala-Ala-Ala	Arsenate (30mM)	59
Gly-Phe		100
Gly-Phe	DCCD (1mM)	108
Gly-Phe	Arsenate (30mM)	46

All peptide concentrations were 100uM

uptake. Arsenate inhibits the uptake of both peptides indicating that their uptake is energised similarly.

Discussion

The inhibition of peptide uptake by arsenate is clear evidence that peptide transport in P. aeruginosa is an active process requiring high energy phosphate bond cleavage to proceed. The lack of inhibition by DCCD indicates that peptide transport is not linked to a membrane proton gradient. Peptide transport in P. aeruginosa would appear to be energised in the same way as that in E. coli. The inhibition of uptake by DNP observed by Cascieri and Mallette (1976a) in P. putida, may indicate a different type of energy coupling for peptide transport from that seen in the other Gram-negative bacteria studied to date, but it may also be caused by a secondary effect of DNP. When the proton gradient is destroyed by DNP, the activity of the membrane bound ATPase which forms the gradient increases, presumably in an attempt to reestablish the gradient. This increased activity causes a reduction in cellular ATP levels which can cause a reduction in ATP dependent activities. DNP has also been shown to inhibit peptide transport in E. coli (Payne and Bell, 1979) although by not as much as arsenate. Experiments with different metabolic inhibitors should determine which mode of energisation is operating in P. putida.

All phosphate bond linked transport systems in *E. coli* are sensitive to osmotic shock, probably because periplasmic binding proteins, needed to shuttle the substrate from the outer membrane to the transport protein at the inner membrane, are lost during shock (Wilson and Smith, 1978). It would be interesting to see if peptide transport in *P. aeruginosa* is similarly "shockable".

5.13 THE EFFECT OF EDTA AND POLYMYXIN B ON PEPTIDE TRANSPORT IN P. AERUGINOSA

Introduction

The outer membrane of Gram-negative bacteria provides an important permeability barrier to many solutes. It has been suggested that the strong resistance of Pseudomonads to a wide range of antibiotics may be due to low permeability of their outer membranes (Brown,1975). As in E. coli, permeation of hydrophilic molecules across the outer membrane of P. aeruginosa is facilitated by porin molecules. Benz and Hancock (1981) showed that the protein F porin of P. aeruginosa formed pores with a diameter of 2.2nm which is significantly larger than the pores formed by OmpF in E. coli (1.4nm). To account for the apparent contradiction of the outer membrane having large pores and being relatively impermeable, Benz and Hancock (1981) suggest that less than 1% of the porin molecules form open pores in vivo . Yoshimura and Nikaido (1982) claim that the outer membrane of P. aeruginosa is significantly less permeable than that of E. coli from the results of assays which measured the rates of hydrolysis of B-lactams and phosphate esters in intact cells and cell sonicates. Yoshimura and Nikaido (1982) have also shown that the values for growth Km of several carbon sources are higher in P. aeruginosa than in E. coli and suggest that

this is further evidence for the outer membrane of P. aeruginosa being a greater permeability barrier than in E. coli. Scudamore and Goldner (1982) claim that the outer membrane contributes little towards the intrinsic antibiotic resistance of P. aeruginosa, a result which would not be expected if the outer membrane was relatively impermeable. Hopefully this apparent contradiction will be clarified by further work.

If the outer membrane does provide a significant barrier to peptide diffusion, the rates of peptide transport measured may be only a fraction of the maximum capacity of the permeases. If the outer membrane permeability barrier could be reduced without affecting the inner membrane then perhaps higher rates of transport might be observed. It has been reported (Brown, 1975) that EDTA and polymyxin B disrupt the outer membrane of P. aeruginosa. At high concentrations both these molecules cause cell lysis, but if a range of concentrations is used it may be possible to specifically damage the outer membrane while leaving the cytoplasmic membrane intact. Several concentrations of both EDTA and polymyxin B were used, and peptide uptake was monitored by both the manual fluorescamine assay and the radioactively labelled peptide assay in an attempt to measure the effect of selective disruption of the outer membrane on peptide transport.

Methods

Cells were harvested as described in section 5.2. The peptide transport asays were performed as described in section 2.5. Cell suspensions were preincubated with EDTA or polymyxin B in potassium phosphate buffer (50mM, pH7.2) for 10min before addition of peptide.

Results

The results of the uptake assays after incubation with either EDTA or polymyxin B are shown in Table 5.10. When measured by the radioactively labelled peptide assay, peptide transport decreases with increasing EDTA concentration until at 100mM EDTA, no uptake is detectable. When measured by the manual fluorescamine assay, transport was affected little by 25mM EDTA but the apparent rate of uptake was increased 400 fold after addition of 250mM EDTA. The effects of polymyxin B addition were similar. When measured by the radioactively labelled peptide assay the transport rate was reduced by increasing polymyxin B concentration until it became undetectable at 100 units ml^{-1} . The fluorescamine assay again showed little effect on peptide transport except at high concentrations when the apparent rate of transport increased dramatically.

Table 5.10. The effect of EDTA and polymyxin B on peptide transport in P. aeruginosa

Peptide	EDTA	Polymyxin B	uptake rate
Ala-Ala-Ala			0.57(R)
Ala-Ala-Ala	5		0.25(R)
Ala-Ala-Ala	20		0.23(R)
Ala-Ala-Ala	100		ND (R)
Ala-Ala-Ala		40	0.39(R)
Ala-Ala-Ala		80	0.19(R)
Ala-Ala-Ala		800	ND (R)
Gly-Phe			0.5 (F)
Gly-Phe	25		0.4 (F)
Gly-Phe	250		180 (F)
Gly-Phe		40	0.4 (F)
Gly-Phe		400	>100 (F)

Rates expresed as nmol peptide min^{-1} mg cellular $protein^{-1}$

ND not detectable

R radioactively labelled peptide assay

F manual fluorescamine assay

Peptides used at 100uM initial concentration

EDTA concentrations in mM

Polymyxin B concentrations in units ml^{-1}

Discussion

The results of the radioactively labelled peptide assays do not show any stimulation of peptide transport by either EDTA or polymyxin B, despite using concentrations of both which have been reported to have effects on the outer membrane. The decline in transport rates measured in this way indicates that the action of both EDTA and polymyxin B is to inhibit apparent peptide transport. This could occur in a number of ways; damage to the outer membrane might lead to loss of a periplasmic binding protein, thus reducing uptake activity or the cytoplasmic membrane might also be damaged producing decreased permease activity and/or leakage of transport products back into the medium, both of which would lead to a decrease in apparent uptake rate. At concentrations of EDTA and polymyxin B which cause loss of over 50% of apparent transport measured by the radioactively labelled peptide assay, rates obtained from the fluorescamine assay show only slight decreases. Bearing in mind the different factors which affect the rates measured by each assay (see section 1.4), this suggests that the decrease in transport rates observed using the radioactively labelled peptide assay may be caused by leakage of amino acid back into the medium. The fluorescence yield of amino acids is relatively low under the conditions used in the fluorescamine assay (see section 1.4) so that the rates measured are

affected little by amino acid exodus, whereas leakage of labelled amino acid into the medium would greatly interfere with the rates obtained from the radioactively labelled peptide assay. At high concentrations of EDTA and polymyxin B the cells are probably lysed, leading to a release of peptidases into the medium. The release of peptidase activity into the medium would cause rapid hydrolysis of the peptide present. This peptide cleavage would be detected using the fluorescamine assay by a rapid decrease in fluorescence yield (indistinguishable from rapid peptide uptake) and the cell lysis would prevent any accumulation of counts inside the cells and abolish apparent uptake shown by the radioactively labelled peptide asssay. In this way the two assays could give such different results from the same effect. Interestingly, the levels of intracellular peptidase activity measured in P. aeruginosa and P. putida (Miller and Becker, 1978; Haas et al., 1981; Cascieri and Mallete, 1976b) correspond closely to the levels of apparent peptide uptake recorded by the fluorescamine assay.

Yoshimura and Nikaido (1982) and Scudamore and Goldner (1982) have recently produced conflicting reports as to the importance of the outer membrane as a diffusion barrier. In this study EDTA and polymyxin B have shown no effects which can be used as evidence for the outer membrane playing an important role in limiting peptide uptake in P. aeruginosa,

although the results indicate that neither compound was specifically disrupting the outer membrane as had been hoped, even though the conditions used were chosen as those most likely to have such an effect.

Further work on the permeability of reconstituted vesicles containing porin protein F and studies of the uptake parameters of other transport systems may lead to a better understanding of the contribution of the outer membrane towards nutrient uptake and antibiotic resistance.

5.14 CONCLUDING DISCUSSION

This study has used direct means to demonstrate peptide uptake in P. aeruginosa. Peptide uptake is a relatively slow process in P. aeruginosa compared with most of the other organisms studied to date, making it more difficult to obtain accurate transport data and limiting the scope of a study such as this.

In summary, the following characteristics of peptide transport in P. aeruginosa may be deduced from the results presented here. Extracellular cleavage of peptides does not play a significant role in transport, and amino acids and peptides are transported via separate systems. Gly-Sar is taken up intact, therefore uptake is not dependent on membrane bound peptidase activity. Peptides at least six amino acid residues in length can be transported. Uptake is probably constitutive and does not appear to be regulated by the nitrogen sources which have been used in this study. There are probably two peptide transport systems, one characterised by a high affinity for Gly-Phe (system A) and one characterised by a high affinity for Ala-Ala-Ala (system B), but both are capable of transporting both di- and oligopeptides. System A is specific for peptides containing L-amino acid residues while system B can tolerate a D-amino acid residue in either the 2nd or 3rd position of a

tripeptide, although the affinity of the permease for such a peptide is much reduced. Both transport systems are coupled to high energy phosphate bond cleavage.

The only previous description of peptide transport in P. aeruginosa (Miller and Becker, 1978) showed, using growth studies, that peptides up to pentapeptides were utilised and that blocking the N-terminus but not the C-terminus prevented uptake, despite the ability to cleave both types intracellularly. Miller and Becker (1978) also found membrane-bound peptidase activity associated with the particulate fraction of lysed cells. This activity was later shown to be associated with the inner rather than the outer membrane fraction (Haas et al., 1981). However intact cells showed no detectable extracellular or periplasmic peptidase activity, therefore if the peptidases are bound to membranes in vivo, they are only accessible at the inner surface of the cytoplasmic cell membrane. Miller and Becker (1978) suggested that hydrolysis might be coupled to peptide uptake as proposed in a model for intestinal absorption by Ugolev et al. (1977). The intact uptake of Gly-Sar indicates that if this does occur it is not the sole mechanism of uptake.

Cascieri and Mallette's study of peptide transport in P. putida (1976a,b) although mainly based on growth tests, shows that uptake is broadly similar to that in P. aeruginosa with

some apparent differences between the two species. Cascieri and Mallette (1976a) reported no competition between di- and oligopeptides and did not detect any competition by Gly-Sar for dipeptide uptake. Uptake of Gly-Gly-Ala was completely inhibited by DNP and tetrapeptides appeared to be near the upper size limit for transport. Many of these dissimilarities may be due to the poor sensitivity of the growth assays used, as early growth studies of peptide uptake in E. coli (Payne and Gilvarg, 1971) gave analogous differences to results from later studies using more direct techniques (Payne, 1980).

Peptide transport in P. aeruginosa and E. coli seem to be qualitatively similar with system A in P. aeruginosa corresponding to the Dpp in E. coli and system B corresponding to the Opp. The only difference found (apart from the obvious difference in rates) was that system B in P. aeruginosa shows some limited specificity towards LDD tripeptides whereas E. coli does not transport LDD tripeptides.

The low rate of transport in P. aeruginosa raises particular difficulties in designing an effective anti-Pseudomonad drug using the "smugglin" concept (Matthews and Payne, 1975b) of joining an otherwise impermeant toxic moiety to a carrier peptide which then carries the moiety

inside the cell where it is released by peptidase activity.

The slow uptake of peptides in Pseudomonads is probably a property of the transport system *per se*, although low outer membrane permeability may also play a part. The contribution of the outer membrane towards permeability is not yet clear and it remains an area which requires further study.

E. coli exists in an environment in which there are alternately high and then low levels of peptides and other nutrients. Under these conditions it is clearly advantageous to transport nutrients rapidly when they are available, therefore *E. coli* has developed high activity peptide transport systems. Pseudomonads, on the other hand, exist in a great variety of environments where they are unlikely to come across high levels of nutrients, but where there is often competition from antibiotic-producing microorganisms. Under these conditions Pseudomonads appear to have developed a relatively impermeable cell envelope and slow transport systems. That the decrease in growth rates produced by slow nutrient uptake is more than compensated for by the ability to compete with other microorganisms, even when they produce antibiotics, is demonstrated by the near ubiquity of Pseudomonads.

6 CONCLUDING REMARKS

In the study of peptide transport in E. coli presented here, mutants defective in dpp, opp and opt have been isolated. The opt gene has been mapped for the first time and has been shown to lie between 84 and 88min on the chromosome. Earlier reports of the locations of the opp and dpp genes (Lenny and Margolin, 1980, Hartmann, 1980) have been confirmed.

These mutants have been used to show that both the Dpp and Opt permeases have wider specificities than has been reported previously (see Payne, 1980 for review), and that considerable overlap occurs between the specificities of the three peptide transport systems. Earlier studies using indirect growth tests had indicated narrower specificities for the permeases but the results presented here demonstrate the necessity of using direct, sensitive transport assays, such as the fluorescamine assay, if transport specificities are to be accurately determined. The range of fluorescence assays now available allows the precise and rapid monitoring of both peptide uptake and amino acid exodus. These assays have been used successfully in a range of organisms including yeasts, Enterobacteria, Pseudomonads and Streptococci, and should enable the peptide transport specificities of clinically important microorganisms to be quickly and

accurately determined. This information is important for the rational design of novel peptide antibiotics. It is also important, in this regard, to have a detailed knowledge of the range of peptidase activities of both the target microorganism and the host tissue to obtain maximum specificity of action.

As the specificities of the three peptide permeases in E. coli are now well characterised, future studies in this organism should attempt to discover the molecular mechanisms of peptide transport. The new techniques which have been developed in molecular genetics should help greatly with this work. The locus coding for Opp in S. typhimurium has already been cloned and is being sequenced (C.F. Higgins, personal communication). When the genes coding for Opp, Dpp and Opt have all been cloned and sequenced, it will be of great interest to determine the degree of homology between the components of the peptide transport systems in a single species and between the transport systems of different species. Presumably those sequences which have been most conserved will be those which are important in the transport process, while those sequences showing differences may be involved in defining the specificities of each system. There may also be an evolutionary relationship between all the ATP-linked transport systems, with transport specificities being mainly a feature of the periplasmic binding proteins

rather than the permease *per se*.

The study of outer membrane protein deficiency on peptide transport showed that the major route for peptide diffusion across the outer membrane was through the porins. Of the three porin types in *E. coli*, OmpF was shown to be more important than either OmpC or PhoE in mediating peptide diffusion. It has been suggested that this could be due to the OmpF pore having a slightly larger internal diameter than the other two pores (Nikaido and Rosenberg, 1983). Other factors, such as the charges on the amino acids lining the pore, may also have a role in determining the pore specificities. What is required is a systematic study of outer membrane permeability using a range of mutants and substrates which have a variety of sizes, charges, hydrophobicities *etc.*. Nikaido and Rosenberg (1983) noted that the pores seemed to be more discriminatory *in vivo* than *in vitro*, possibly because interactions with other outer membrane components are necessary for normal pore function. This being the case, the systematic study should use intact cells. Monitoring peptide transport kinetics as described in Chapter 4 allows the use of a wider range of sizes, charges *etc.* than any of the other substrates used so far e.g. sugars, B-lactams and would seem to be ideal for such a study.

The loss of the OmpA protein has been shown to have a marked effect on peptide transport in three different strains of E. coli K12. It seems unlikely that the OmpA protein forms a pore itself as it showed no pore-forming activity in the liposome assay (Nakae, 1976a), although it has not been shown that OmpA does not form pores in vivo. As the porins are only active in the presence of LPS, the loss of OmpA might decrease the activity of the porin channnels by disrupting the interactions between porins and the LPS. Further work on the localisation and interactions between outer membrane components in the absence of OmpA is required to clarify the exact role of the OmpA protein in the pore-forming process.

An understanding of how solutes diffuse through the porins and how to maximise the rate of diffusion across the outer membrane would be useful in the design of most antibiotics as almost all toxic molecules must penetrate the outer membrane to reach their site of action.

The first evidence for intact peptide uptake in P. aeruginosa has been presented in Chapter 5. The use of direct fluorescence and radioactively-labelled peptide uptake assays has shown that uptake in P. aeruginosa is similar to uptake in E. coli with regard to energisation and stereospecificity. There appears to be at least two peptide

permeases in P. aeruginosa which are qualitatively similar to the Dpp and Opp in E. coli, although further competition studies using different peptides should be undertaken to confirm this.

The major difference between the peptide uptake systems in the two species is the rate at which transport occurs, as peptide transport is almost two orders of magnitude slower in P. aeruginosa than in E. coli. As Pseudomonads are renowned for their ability to utilise a wide range of potential nutrients, it might have been expected that a higher rate of transport could be induced under suitable growth conditions. A range of nitrogen sources was used, but peptide transport remained at its low constitutive level. However even the low levels of transport measured in this study, which are similar to the rates of amino acid transport previously determined (Kay and Gronlund, 1969a,b), may be sufficient to supply the cells requirement for amino acids under natural growth conditions, even assuming none of the amino acid was synthesised from other N-sources, as the following calculation shows. 1mg of cellular material would have to be synthesised in one generation, in a culture containing 1mg of cells per ml. Assuming that half of the cell mass is protein, then 500ug of protein would have to be synthesised. If there are 20 protein amino acids, then 25ug of each amino acid will be required for one cell generation. Therefore

250nmol of each amino acid or 100nmol of a mixture of di- and tripeptides would have to be transported into the cell per generation to satisfy the amino acid requirement (assuming an average amino acid molecular weight of 100). If the rate of peptide uptake was 0.5nmol min^{-1} mg^{-1}, enough amino acid for one generation could be transported in 200min. The average generation time in a natural environment is likely to be slower than this, therefore both peptide and amino acid transport are likely to be sufficiently rapid to meet the organism's needs.

The resistance of P. aeruginosa to many antibiotics has been ascribed to a relatively impermeable outer membrane. Early investigations (Hancock and Nikaido, 1978) of the permeability of the outer membrane reported an exclusion limit for molecules above a molecular weight of 6,000 (compared with an exclusion limit of 600 in E. coli). Benz and Hancock (1981) reported that the porin protein F had a pore size of 2.2nm (compared with 1.4nm for OmpF in E. coli), but to explain the low membrane permeability in the presence of such large pores, they suggested that only a small proportion of the pores in P. aeruginosa were open in vivo. Recently, Caulcott et al., (1984) have reported an apparent exclusion limit for molecules of molecular weight over 400. The results of peptide transport studies (Miller and Becker, 1978; Chapter 5, this study) have shown that penta-Met

(molecular weight 673) and hexa-Ala (molecular weight 444) are transported into P. aeruginosa. Both of these peptides are larger than the exclusion limit proposed by Caulcott et al. (1984). Further experiments similar to those being used to characterise the pore types in E. coli are required to determine the nature of the outer membrane permeability barrier in P. aeruginosa.

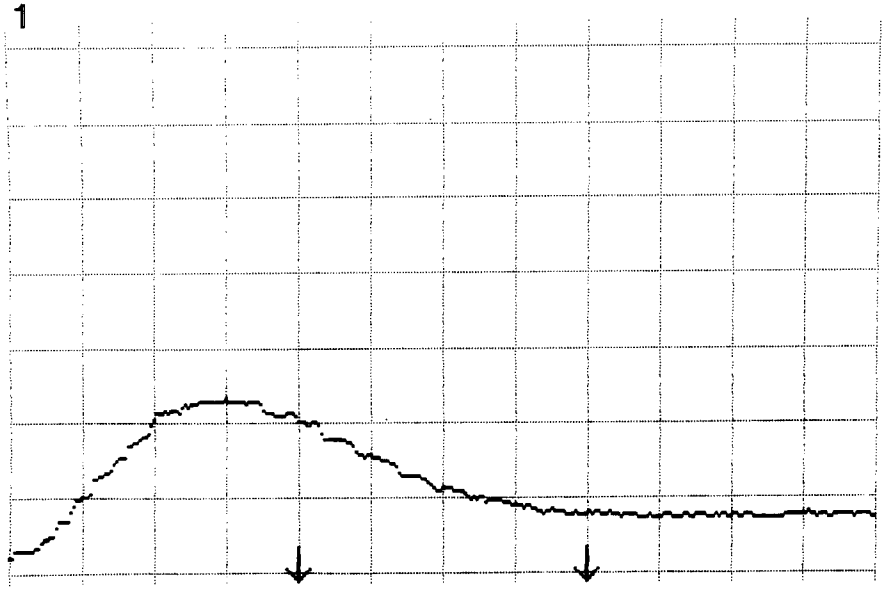
APPENDIX A

An example of the data gathering and processing procedure used in the calculation of uptake kinetics in Chapter 5 is shown here. Graphs 1, 2 and 3 (see Plates A.1 and A.2) are traces of the uptake of Ala-Ala by strain H1. 1 shows an assay with an initial peptide concentration of 100uM, 2 shows an assay with an initial peptide concentration of 200uM and 3 shows an assay with an initial peptide concentration of 500uM. Graphs 1a, 2a and 3a (see Plates A.1 and A.2) are the traces shown in 1, 2 and 3 respectively, after the data has been corrected for amino acid exodus, as described in section 4.5. Graph 4 shows the best fit Michaelis Menten parabola calculated from the data using the Micmen program. Graph 5 is a representation of the 95% confidence limits which can be placed on the estimates for Km and Vmax, prepared by the subroutine contour of the Micmen program. If two different contours are overlaid, it can be seen whether the estimates are significantly different from each other or not.

Plates A.1 and A.2

An example of the peptide uptake data gathering and processing used t
calculate the Michaelis-Menten kinetic parameters in Chapter 4. See
text for description of each trace.

1



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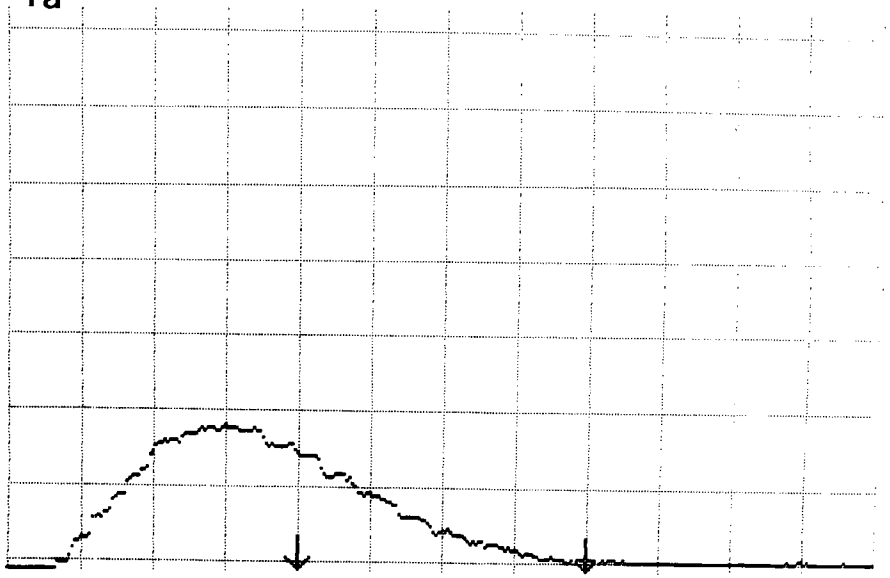

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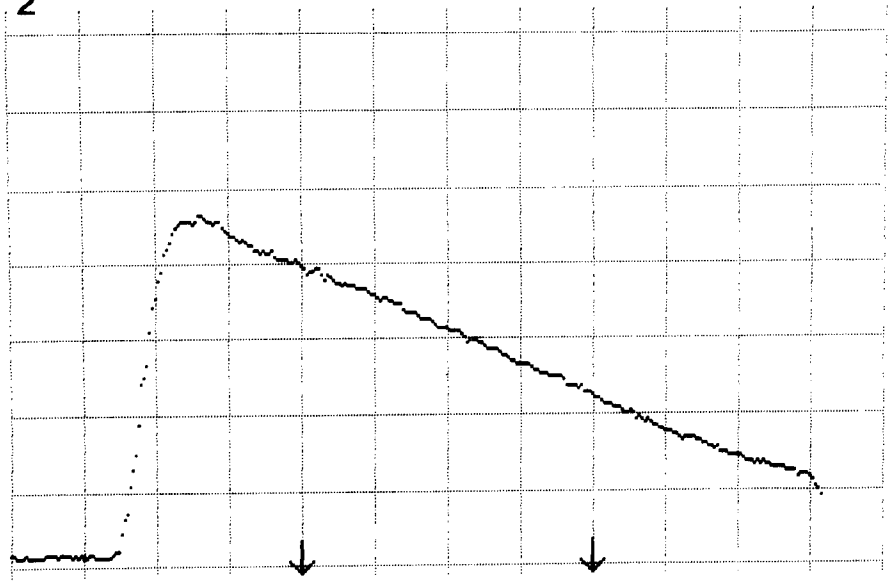

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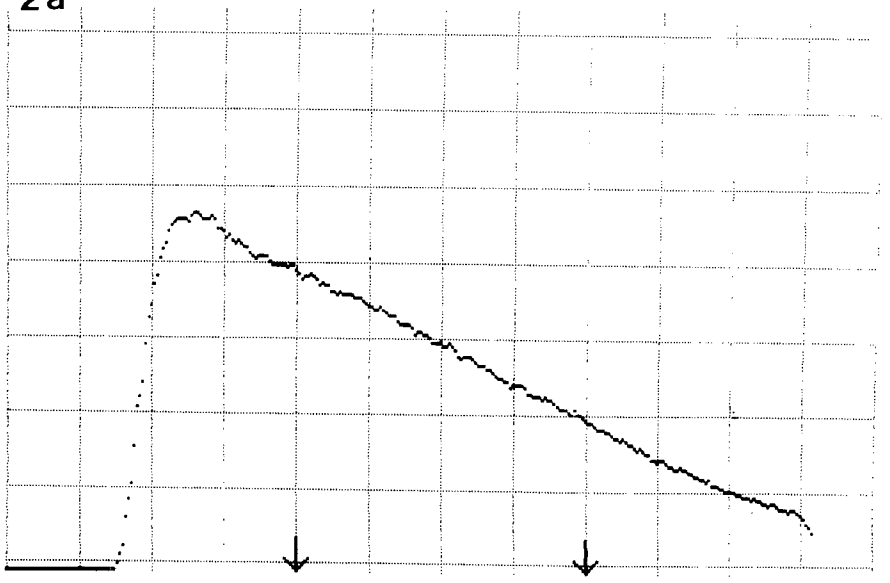

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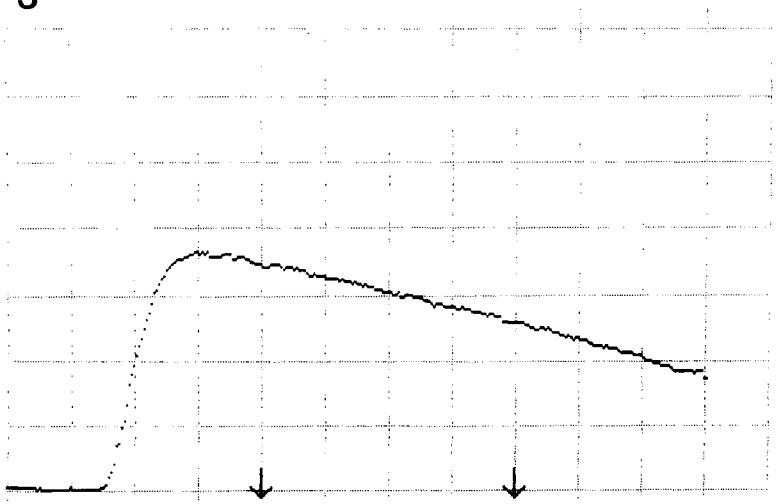

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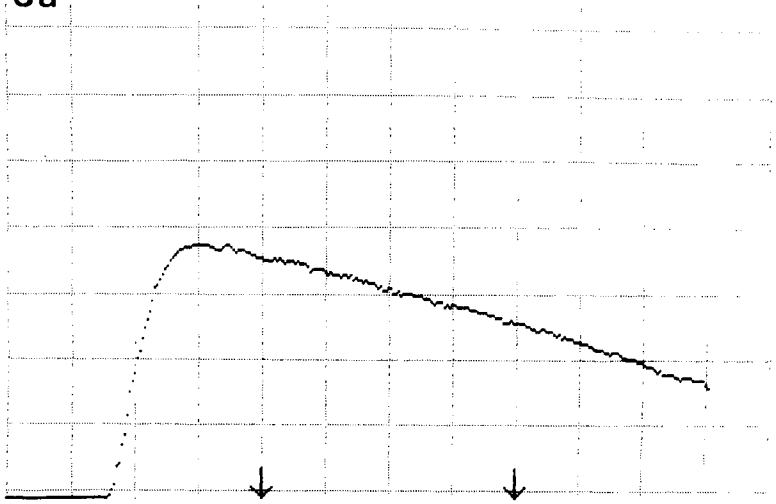

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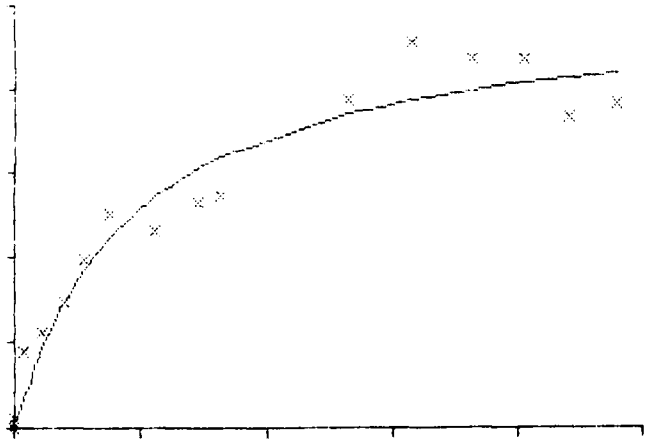

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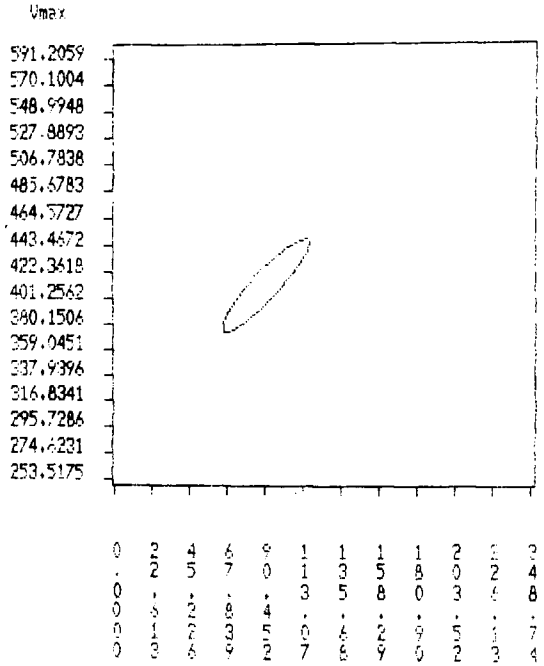
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485.6783
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443.4672
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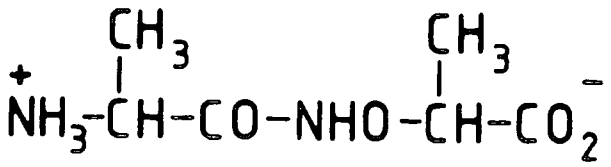
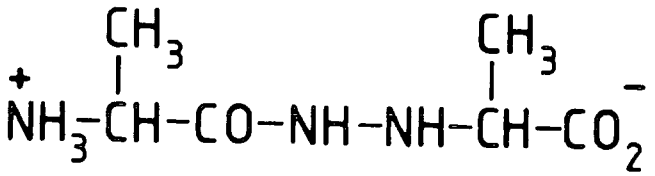
401.2562
380.1506
359.0451
337.9396
316.8341
295.7286
274.6231
253.5175

APPENDIX B

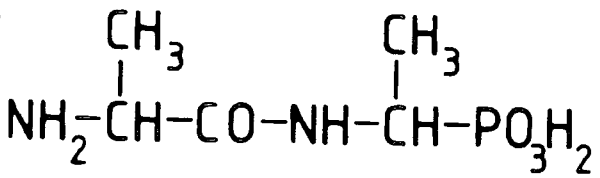
Most of the peptide mimetic analogues used in this study are of three types, aminoxy peptides, hydrazino peptides and phosphono peptides (for an explanation of the nomenclature see Morley et al. (1983). The peptides shown here are all L-Ala-L-Ala derivatives although any other amino acid residues could be substituted. Peptide A (see Figure B.1) is Ala-aminoxyAla, which has an extra oxygen inserted into the peptide backbone, peptide B, is Ala-hydrazinoAla, which has an extra NH inserted into the peptide backbone and peptide C is Ala-Alaphosphonic acid, which has a phosphonic acid group substituting for the C-terminal carboxyl group.

A 

Ala-aminoxyAla

B 

Ala-hydrazinoAla

C 

Ala-Alaphosphonic acid

Figure B.1 Chemical structures of peptide mimetic antibacterial agents.

Appendix C

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